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Identification of Six Single-Strand Initiation (*ssi*) Signals for Priming of DNA Replication in Various Plasmids

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Abstract: Using a mutant M13 phage derivative lacking a great part of the complementary strand synthesis origin, we identified six single-strand initiation (*ssi*) signals for DNA replication in pACYC184, pLG214, pGKV 21, and pDPT270 plasmids, and named them *ssi*A_{YC}, *ssi*A_{LG}, *ssi*B_{LG}, *ssi*A_{KV}, *ssi*A_{PT}, and *ssi*B_{PT}, respectively. Two of them were from pDPT270, one from downstream the *ori* of pACYC184, two from pLG214, one from upstream the plus origin of pGKV21. Introduction of these *ssi* signals into the deleted *ori*$_c$ site of a mutant filamentous M13 phage (M13Δlac182) resulted in the restoration of growth activity of this phage. These *ssi* signals were classified into a number of groups on the basis of sequence similarity. *ssi*A_{YC} and *ssi*A_{LG} show extensive sequence homology to the n′-site (primosome assembly sites) of ColE1, whereas *ssi*B_{PT} is homologous to the n′-site of ΦX174. *ssi*A_{PT} belongs to G4-type *ssi* signals which require only *dnaG* primase and SSB protein for the priming of replication. In addition, possible biological roles of these *ssi* signals are discussed.

Key words: dnaG, n′-site, replication, *ssi* signal.

DNA replication is an essential phenomenon for cell division and most precisely regulated during its initiation process. Plasmids are useful model systems for the analysis of the initiation mechanisms of DNA replication (Kornberg, 1992). An *ssi* signal is defined as a specific nucleotide sequence on the ss DNA template directing the priming of the complementary DNA strand synthesis. Since priming of both the continuous leading strand and the discontinuous lagging strand syntheses takes place on the ss DNA templates, studies on *ssi* signals are particularly significant for understanding initiation events. The origins of complementary DNA strand synthesis of ss DNA phages are typically *ssi* signals (Kaguni and Ray, 1979; Strathearn *et al.*, 1984; Sakai *et al.*, 1987). Most of the plasmids examined thus far have one or more *ssi* signals. The *ssi* signals can be classified into several types in terms of their functional characteristics. First, some *ssi* signals from R1 (Bahk *et al.*, 1988; Masai and Arai, 1989), R6K, and F (Masai *et al.*, 1990a), are G4-type *ssi* signals which require only *dnaG* primase for the priming in the presence of SSB (single-strand binding protein). Second, *ssi* signals from plasmid ColE1 are n′-sites (primosome assembly sites), such as ΦX174-type *ssi* signals. These ΦX174-type *ssi* signals direct the multiple initiation of lagging strand synthesis, and are the assembly site of an n′-dependent primosome, which is responsible both for the translocation and for the repeated priming in Okazaki fragment synthesis (Minden and Marians, 1985; Masai and Arai, 1988; Lee and Marians, 1989). Third, one of the *ssi* signals from plasmid R6K directs the assembly of the DnaA, DnaB, and DnaC proteins of *E. coli*. The functional protein complex is referred to as an ABC-primosome and is mobile on the template DNA directing multiple initiation events. This ABC-type *ssi* signal can replace the ΦX174-type *ssi* signal and direct the initiation of the lagging strand synthesis, suggesting a possible role of ABC-primosome in the lagging strand synthesis and duplex unwinding (Masai *et al.*, 1990b). Fourth, the function of two *ssi* signals in the broad host range plasmid RSF 1010 depends on the plasmid-encoded RepB′ protein *in vivo* (Honda *et al.*, 1989). RepB′ protein functions in the *oriV* region of the plasmid RSF1010 (Honda *et al.*, 1989), and is responsible for the initiation of DNA strand synthesis on both strands. Exhaustive attempts to elucidate their roles in plasmid DNA replication are in progress. Some *ssi* signals are believed to be involved in the initiation of vegetative DNA replication

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of plasmids including the leading strand and the lagging strand syntheses (Masai *et al.*, 1990a), although they are not located in the minimum origin of DNA replication.

The aim of the present study is to isolate and characterize various *ssi* signals from the pACYC184, pLG 214, pGKV21, and pDPT270 plasmids and to discuss their biological roles in RNA priming of the lagging and leading strand syntheses of DNA replication.

Materials and Methods

Bacterial strains, phages, and plasmids

The *E. coli* strains, bacteriophages, and plasmids used in this study are listed in Table 1. *E. coli* strains, TG1 and JM101, were used as host bacteria for filamentous phage vectors. The filamentous phage vector M13Δlac 182, an *ssi*-probe vector, is a minus origin-deleted mutant M13 phage vector.

Enzymes and chemicals

Restriction enzymes and DNA-modifying enzymes were purchased from Bethesda Research Laboratories (Grand Island, USA) and Boeringer Mannheim (Mannheim, Germany). IPTG, X-gal, and other chemicals were purchased from Sigma Chemicals Co (St. Louis, USA). All enzymes were used according to the manufacturer's directions.

Phage growth assay

Growth assay of phages was done as previously described in Bahk *et al.* (1990). Bacterial growth after phage infection was carried out at 37℃ in 2X YT media.

Results and Discussion

Two *ssi* signals of pDPT270 function each with a different mode in the priming process

Plasmid pDPT270 is derived from the miniplasmid pDPT101, and so contains the replicon of an R plasmid NR1 copy number mutant pRR21 (Taylor *et al.*, 1977). Primary detection for clones containing SSI activity from the pDPT270 plasmid was done following the plaque morphology procedure, using a mutant M13 phage vector (M13Δlac182) which forms small turbid plaques because it lacks a great part of the minus origin for complementary DNA strand synthesis. When a correct *ssi* signal is inserted into the M13Δlac182, the recombinant phage can form larger and clearer plaques than M13Δlac182 can. First, the plasmid pDPT 270 DNA was cleaved with *Alu*I, *Rsa*I, and *Hae*III, and then introduced into the *Sma*I site of M13Δlac182 (an *ssi*-probe vector). We obtained some positive clones containing SSI activity. Through repeated subcloning, we finally found that at least two *ssi* signals were present in pDPT270. One was in the 170 nt *Eco*RI-*Acc*II segment, and the other was in the 125 nt *Dra*I-*Alu*I segment, which were designated as *ssi*A_{PT} and *ssi*B_{PT}, respectively (Fig. 1). The *ssi*A_{PT} is located in the region downstream from *oriR*. Interestingly, a computer-assisted homology search of the nucleotide sequence using the GCG package program with the GenBank database showed that the *ssi*A_{PT} was highly homologous with the G4-type *ssi* signals, such as those of F and R6K etc., which direct the initiation of the leading strand synthesis (Fig. 5). Plasmid pDPT270 is closely related to plasmid R1 and is thought to have the same replication mechanism as plasmid R1 (Miyazaki *et al.*, 1988). In the case of plasmid R1, it was previously known that the nucleotide sequence on the lagging strand, nearly 400 nt downstream from *oriR*, could determine the SSI function and direct the initiation of leading strand synthesis. Similarly, in the case of plasmid pDPT 270, the *ssi*A_{PT} is nearly 400 nt downstream from *oriR* and is on the lower strand, which corresponds

Table 1. *E. coli* strains, bacteriophages and plasmids used in this study

Strains	Relevant properties	Sources
***E. coli* strains**		
TG1	*supE hsd*Δ5 *thi* Δ(*lac-pro*) F'[*tra*D36 *pro*A^+B^+*lacI*^{q}*lacZ*ΔM15]	Gibson (1984)
JM101	*supE thi* Δ(*lac-proAB*) F'[*tra*D36 *pro*A^+B^- *lacI*^{q}*lacZ*ΔM15]	Messing (1979)
Bacteriophages		
M13mp18	Filamentous single strand DNA M13 phage vector	Messing (1983)
M13Δlac182	M13 phage vector lacking its origin of complementary strand synthesis	Kim *et al.* (1981)
Plasmids		
pDPT270	NR1 copy number mutant pRR21 replicon	Taylor *et al.* (1979)
pACYC184	p15A replicon	Chang and Cohen (1978)
pLG214	pBR325 replicon, *sog* gene	Wilkins and Boulnois (1981)
pGKV21	Lactococcal plasmid pWV01 replicon	Van der Vossen *et al.* (1985)

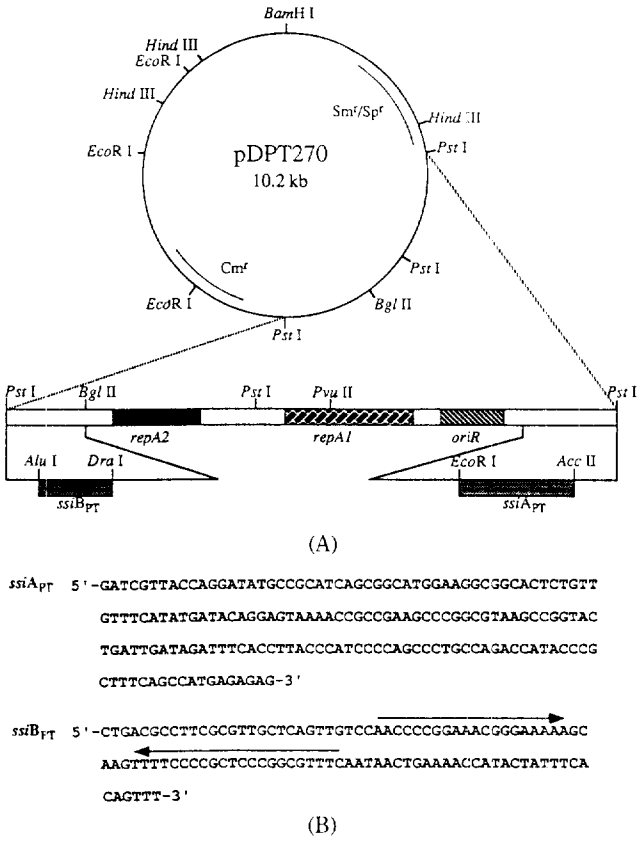

Fig. 1. Location and nucleotide sequence of the *ssi* signals of pDPT270. A) Physical map of pDPT270 and of a *Pst*I fragment containing the *ssi* signals, which are based on results of Taylor *et al.* (1979). *oriR* indicates replication origin. *repA1* and *repA2* indicate genes required for replication. B) Nucleotide sequences of the *ssi* signals in pDPT270. The inverted repeat sequence is indicated by arrows over the nucleotide sequences.

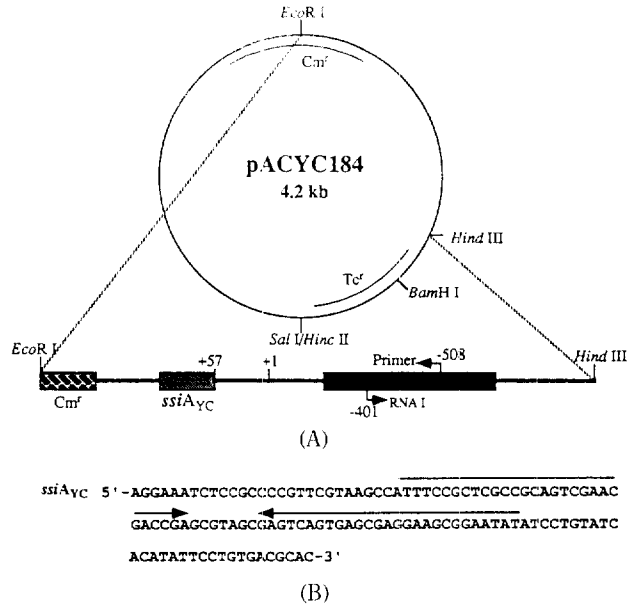

Fig. 2. Location and nucleotide sequence of the *ssi* signal of pACYC184. A) Physical map of pACYC184 and of the *Eco*RI-*Hin*dIII fragment containing the *ssi* signal. The +1 indicates the first of three consecutive bases which serve as the origin of DNA replication. Arrows indicate the start site and direction for transcription of primer and RNAI. B) Nucleotide sequence of the *ssi*A_{YC} segment illustrated in panel A.

to the lagging strand in plasmid R1. Judging from the above results, it is reasonable that *ssi*A_{PT} functions in priming the leading strand synthesis of pDPT270 plasmid. The role of *ssi*B_{PT}, which is located just outside the basic replicon of the pDPT270 plasmid, is not clear. Although modes of priming on *ssi*B_{PT} might not be predicted by its location, sequence homology studies suggested that *ssi*B_{PT} could initiate replication according to a ΦX174 type priming mechanism (Fig. 5).

A 119 nt *ssi* signal of pACYC184 could efficiently function in the lagging strand synthesis

Plasmid pACYC184, a derivative of the plasmid p15 A of *E. coli*, is a 4.2 kb relaxed copy number plasmid conferring resistance to chloramphenicol and tetracycline (Chang and Cohen, 1978). To identify an *ssi* signal in pACYC184, the plasmid DNA of pACYC184 was digested with various restriction enzymes, and then inserted into the *ssi*-probe vector (M13Δlac182). Finally, we obtained a clear plaque-forming phage carrying only the 119 nt *Bst*NI-*Hpa*II segment, conferring SSI activity, which represented the nearly minimal region (Fig. 2). In the 119 nt *ssi* signal, two kinds of consensus sequences, 5'-CGCTCGCCGCAG-3' and 5'-GAAG-CGG-3', were conserved. The 7 nt stretch, 5'-GAAG-CGG-3', had been known as the n' protein recognition sites (Van der Ende *et al.*, 1983). Moreover, the 12 nt stretch, 5'-CGCTCGCCGCAG-3', had been known as the *dnaB*, *dnaC* and *dnaG*-dependent initiation signal. The *ssi* signal carried by this phage was named *ssi*A_{YC}. In this region, we found a potential stem-loop structure. In a study using site-directed mutagenesis, we have shown that the stem region and 3'-site region of the stem-loop structure are essential for SSI function, but mutations of the 5'-site of the stem-loop structure showed slightly depressed SSI activities (Jeong *et al.*, 1994a). The *ssi*A_{YC} is located 57 nt downstream from the *ori* of the leading strand for DNA replication (Fig. 2). The direction of chain elongation of the DNA strand by the SSI function is opposite to that of the leading strand, and we previously reported in another paper (Jeong *et al.*, 1994a) that many kinds of deletions on the pACYC184 genome lead to reducing the copy number of the plasmid. On the other hand, introduction of a ΦX174 n'-site into the deleted-*ssi* site of pACYC 184 could complement the defect in replication of *ssi*-deleted plasmids. Judging from these results, it could be concluded that the 119 nt ssi signal from pACYC 184 must function efficiently in the lagging strand syn-

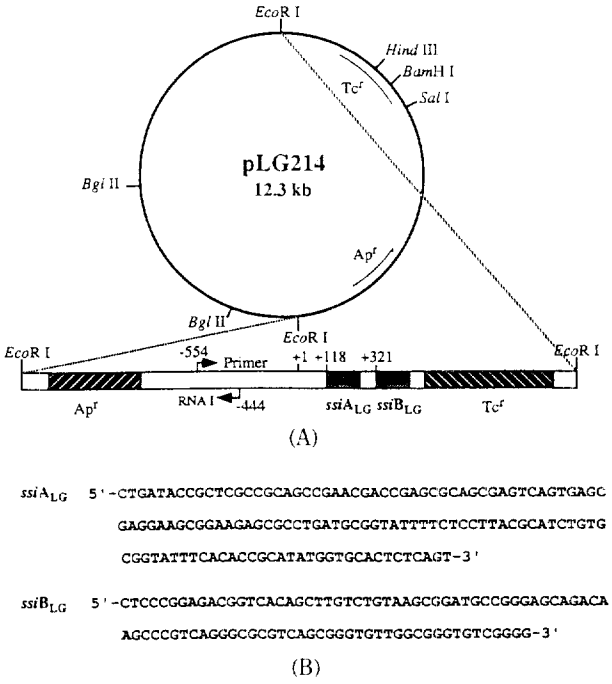

Fig. 3. Location and nucleotide sequence of the *ssi* signals of pLG214. A) Physical map is based on results of Wilkins and Boulnois, (1981). The lower linearized box indicates the map of pBR325 generated by *Eco*RI digestion. B) Nucleotide sequences of ssi signals of pLG214.

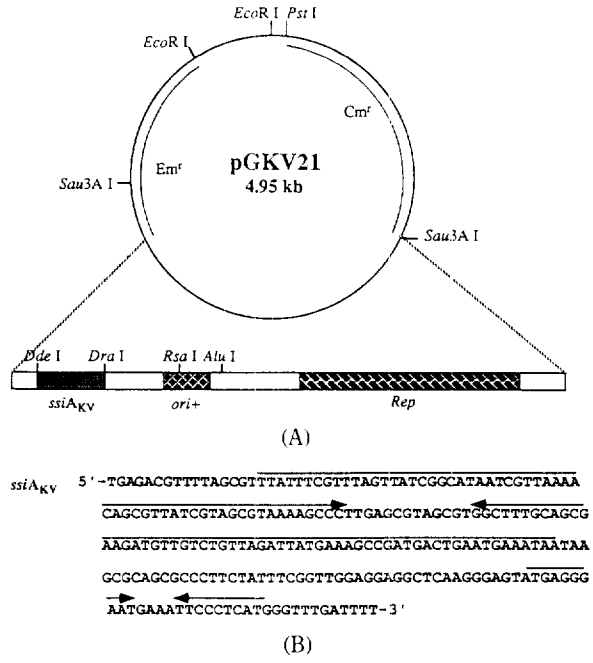

Fig. 4. Location and nucleotide sequence of the *ssi* signal of pGKV21. A) Physical map of pGKV21 is based on the data reported by Van der Vossen *et al.* (1985). The lower linearized box indicates the map of pWV01. The Rep and *ori*+ represented the region encoding Rep protein and plus origin, respectively. B) Nucleotide sequence of an *ssi* signal in pGKV21. The sequence is a 229 nt of *Dde*I-*Dra*I segment, which has the possibility of forming two stem-loop structure. The expected palindromic sequences are indicated by arrows over the nucleotide sequences.

thesis.

Two *ssi* signals of pLG214 contain portions identical with the n′-sites of pBR322 and ColE1 plasmids

Plasmid pLG214 is a derivative of plasmid pBR325 containing the *sog* gene of Col*drd-1* (Wilkins and Boulnois, 1981). The *sog* gene is a DNA primase encoded by IncIα plasmid and promotes efficient DNA replication in a primase-defective mutant of *E. coli*. First, we attempted to detect the *cis*-element (*ssi* signal) affected by the sog gene of pLG214. However, the *ssi* signal was not detected from the DNA fragments of this plasmid. Instead, we detected two *ssi* signals in the DNA fragments of pBR325. One was in the 134 nt *Alu*I-*Rsa*I segment, and the other was in the 92 nt *Alu*I-*Hha*I segment, which were designated as *ssi*A_{LG} and *ssi*B_{LG}, respectively (Fig. 3). These two *ssi* signals of pLG214 are identical to n′-sites of pBR322 and ColE1, which had already been reported (Nomura and Ray, 1980; Nomura *et al.*, 1982; Zipursky and Marians, 1981). These n′-sites were shown to stimulate DNA-dependent ATPase activity of protein n′ (Shlomai and Kornberg, 1980; Nomura *et al.*, 1982) and promoted the rifampicin-resistant conversion of ss DNA into the duplex form when introduced into filamentous single-stranded phage DNA (Nomura and Ray, 1980; Nomura *et al.*, 1982; Marians *et al.*, 1982).

In the pBR322, since one of the n′-sites (L-n′ site) is located on a lagging strand template, downstream the origin of this plasmid, the L-n′ site has been considered as an origin of lagging strand DNA synthesis (Nomura and Ray, 1980; Nomura *et al.*, 1982; Marians *et al.*, 1982). Masai and Arai (1988) reported that an efficient replication of pBR322 requires the presence of an n′ site on the L-strand, although the effect of the n′ site might be not so significant. Further analysis of the function of *ssi*A (ColE1), located on the lagging strand template downstream of the *ori*, demonstrated the crucial role of the primosome assembled at this n′-pas for efficient lagging strand synthesis *in vitro* (Miyazaki *et al.*, 1988; Masai *et al.*, 1988).

The stem-loop II structure within an *ssi* signal of pGKV21 is important for efficient *ssi* signal activity

Plasmid pGKV21 is a derivative of the lactococcal plasmid pWV01 (Kok *et al.*, 1984; Van der Vossen *et al.*, 1985) which is a broad host range plasmid being able to replicate in gram-positive as well as gram-negative bacteria (Leenhouts *et al.*, 1991). Through repeated subcloning experiments, we obtained large and clear plaques of the recombinant phage carrying only

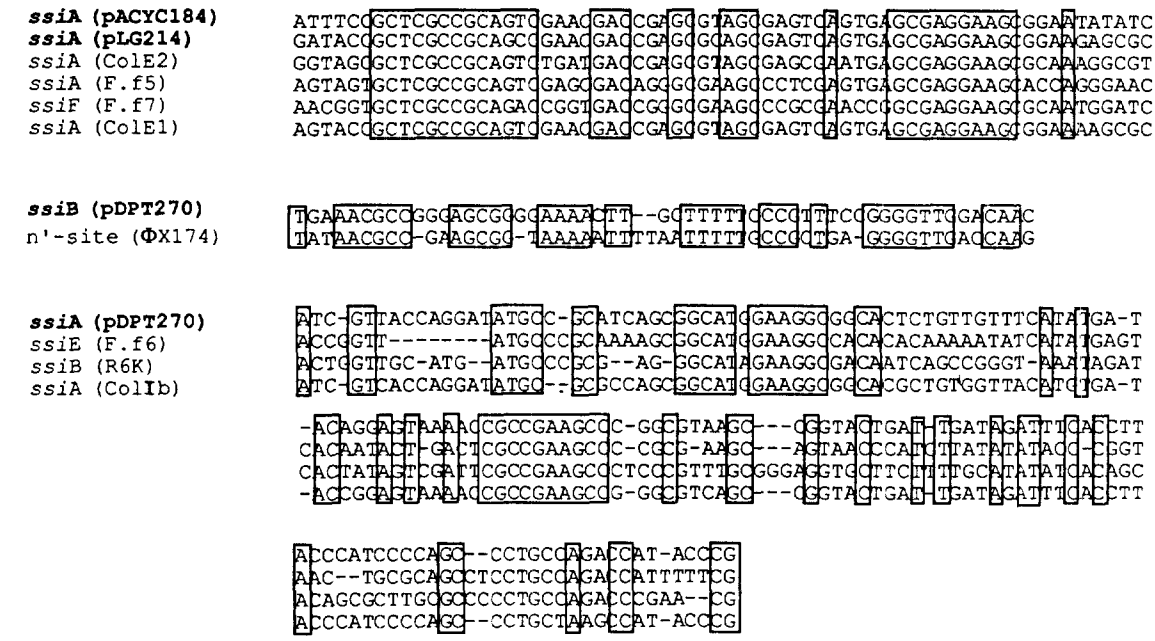

Fig. 5. Alignment of many *ssi* signals obtained from plasmids and bacteriophages. Three groups which show sequence homology were aligned. Conserved sequences are boxed. Dashes indicate gaps introduced to obtain maximal alignment.

229 nt of the *Dra*I-*Dde*I segment into the *Sma*I site of M13Δlac182 (Fig. 4). However, replication activity of the *ssi* signal in pGKV21 maintained a level lower than those of *E. coli* plasmids. It seemed that the priming efficiency of pGKV21 isolated from gram-positive bacteria might be lower than those of other *E. coli* plasmids. This *ssi* signal could form two potential stem-loop structures, stem-loop I and II. Stem loop I showed some characteristics similar to the minus origins of other plasmids from gram-positive bacteria. For example, the consensus sequence, 5′-TAGCGT-3′, presented in the loop of *palA*-type minus origins, was also located in the loop portion of the stem-loop I structure. In addition, this structure showed some sequence similarities with the complementary strand synthesis origin of ΦX 174. However, stem loop II had no apparent sequence similarities among other *ssi* signals. We had previously showed that the stem-loop II region is required for full activity through Bal 31 nuclease deletion mutagenesis (Jeong *et al.*, 1994b). This *ssi* signal was located near 109 nt upstream the nick site of the putative plus origin, and the 229 nt DNA stretch of pGKV21 was orientation-dependent for SSI activity, that is, in the direction of *Dde*I to *Dra*I. Based on the location of the *ssi* signal relative to those of the known plus origins, and from the direction of SSI activity, we propose that the *ssi* signal could function as a minus origin of plasmid pGKV21.

Nucleotide sequence homologies among various *ssi* signals

The six *ssi* signals described here were isolated from four plasmid genomes using a plaque morphology assay. The *ssi* signals can be divided into three groups based on sequence homology (Fig. 5). The n′-site (primosome assembly site) can be divided into at least two types, that is, the n′-pas of ΦX174 and n′-pasA (ColE1). DNA homology studies showed that $ssiB_{PT}$ belongs to ΦX174 type, and $ssiA_{YC}$ and $ssiA_{LG}$ belong to ColE1 type. $ssiA_{PT}$ showed extensive sequence homology to the *ssi*E (F-f6), *ssi*B (R6K) and *ssi*A (ColIb). They have conserved sequence motifs different from the ΦX174 type, and are suggested to be able to function with a G4-type mechanism. Although the priming mode of $ssiB_{LG}$ might not be predicted from sequence homology, *in vitro* studies showed that it made use of the ΦX 174 type priming mechanism (Masai *et al.*, 1990). The $ssiA_{KV}$ had not shown sequence similarity among the minus origins of other plasmids from gram-positive bacteria, but this region was homologous to the origin of complementary strand synthesis of ΦX 174.

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