***Escherichia coli* PriA protein is essential for inducible and constitutive stable DNA replication**

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Under certain conditions, *Escherichia coli* cells exhibit either of two altered modes of chromosomal DNA replication. These are inducible stable DNA replication (iSDR), seen in SOS-induced cells, and constitutive stable DNA replication (cSDR), seen in *rnhA* mutants. Both iSDR and cSDR can continue to occur in the absence of protein synthesis. They are dependent on RecA protein, but do not require DnaA protein or the *oriC* site. Here we report the requirement for PriA, a protein essential for assembly of the ϕ X174-type primosome, for both iSDR and cSDR. In *priA1(Null)::kan* mutant cells, iSDR is not observed after induction by thymine starvation. Replication from one of the origins (*oriM1*) specific to iSDR is greatly reduced by the *priA1::kan* mutation. cSDR in *rnhA224* mutant cells deficient in RNase HI is also completely abolished by the same *priA* mutation. In both cases, SDR is restored by introduction of a plasmid carrying a wild-type *priA* gene. Furthermore, the viability of an *rnhA::cat dnaA46* strain is lost at 42°C upon inactivation of the *priA* gene, indicating the lethal effect of *priA* inactivation on those cells whose viability depends on cSDR. These results demonstrate that a function of PriA protein is essential for iSDR and cSDR and suggest the involvement of the PriA-dependent ϕ X174-type primosome in these DnaA/*oriC*-independent pathways of chromosome replication. Whereas ColE1-type plasmids, known to be independent of DnaA, absolutely require PriA function for replication, DnaA-dependent plasmid replicons such as pSC101, F, R6K, Rts1 and RK2 are able to transform and to be maintained in the *priA1::kan* strain. We propose that bacterial replicons can be classified into two categories, one that depends on the DnaA-dependent primosome and another that relies on the PriA-dependent primosome.
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Introduction

Initiation of DNA replication requires strand opening or melting of duplex DNA prior to assembly of a 'primosome'

responsible for primer RNA synthesis and continuous unwinding of double-stranded DNA at the replication fork (Bramhill and Kornberg, 1988; Umek and Kowalski, 1988; Umek *et al.*, 1989; Asai and Kogoma, 1994). Under normal growth conditions, *Escherichia coli* cells initiate chromosome replication at a unique site, *oriC*, after strand opening is effected by the specific binding of DnaA protein to the *oriC* sequences (Kornberg and Baker, 1991). Subsequently, the DnaB helicase is loaded onto DNA at the melted region leading to assembly of the 'oriC primosome'. The primosome further unwinds the duplex for replisome assembly. The DnaG primase, in association with DnaB, synthesizes RNA primers for DNA chain elongation at the replication forks (Bramhill and Kornberg, 1988).

When DNA damage or DNA synthesis inhibition induces the SOS response or when a mutation in the *rnhA* gene decreases the cellular RNase HI activity, chromosomal replication becomes independent of the *oriC* site and DnaA protein (for a review see Asai and Kogoma, 1994). These altered modes of replication, called inducible or constitutive stable DNA replication (iSDR or cSDR) respectively, require RecA protein (Lark and Lark, 1978). Whereas ongoing protein synthesis is dispensable for both types of SDR, transcription is essential for cSDR but not for iSDR. Thus, iSDR is resistant both to chloramphenicol (Cam), an inhibitor of protein synthesis, and to rifampicin (Rif), a transcription inhibitor, while cSDR is resistant only to Cam. Genetic analyses have indicated that the strand opening essential for initiation of iSDR is achieved by formation of D-loops, which are created by the actions of the RecA and RecBC proteins (Magee and Kogoma, 1990; Asai *et al.*, 1993). On the other hand, cSDR is proposed to be initiated from an R-loop created by a transcript which hybridizes to the coding strand, displacing the opposite strand. R-loops are normally removed by RNase HI, but in its absence they are stabilized and function as origins of replication (von Meyenburg *et al.*, 1987). Inasmuch as neither DnaA protein nor *oriC* is required for SDR, it has been predicted that primosomes that drive replication forks during SDR may be distinct from the *oriC* primosome (Masai and Arai, 1988b).

The ϕ X174-type primosome was originally discovered from enzymatic studies of the conversion of single-stranded circular (SS) to replicative form (RF) DNA in phage ϕ X174. Later, it was demonstrated, both *in vitro* (Minden and Marians, 1985; Masai and Arai, 1988a) and *in vivo* (Masai and Arai, 1989a), that replication of ColE1-type plasmids depends on the ϕ X174-type primosome, which involves several *E.coli* replication proteins. PriA protein specifically binds to a hairpin structure called n'-pas (primosome assembly site) on single-stranded DNA binding protein (SSB)-coated ϕ X174 DNA. PriB (protein n) then binds to the PriA–DNA complex. A hexameric

DnaB helicase is delivered from a DnaB–DnaC heterohexamers to the PriA–PriB–DNA complex by the action of DnaT (protein i). PriC (protein n') is also thought to participate in this process (Allen and Kornberg, 1993). Subsequently, presumably through interaction with DnaB, the DnaG primase participates in the complex, completing assembly of a primosome.

Isolation of *n'-pas* from dispersed locations of other plasmids, such as F and R100 (Nomura *et al.*, 1991), has suggested possible functions of the ϕ X174-type primosome in normal and transfer replication of these plasmids, although its involvement has not been demonstrated in these instances. The *priA* gene encoding PriA (protein n') has been cloned (Lee *et al.*, 1990; Nurse *et al.*, 1990). Inactivation of the *priA* gene by deletion–insertion does not kill the cell (Lee and Kornberg, 1991; Nurse *et al.*, 1991), indicating that PriA protein is not absolutely essential for viability under normal growth conditions, although the mutation substantially reduces growth rate and greatly increases UV sensitivity. This result implies that the ϕ X174-type primosome may be dispensable for normal chromosomal replication initiating at *oriC*.

We previously reported that protein i (encoded by the *dnaT* gene) and DnaC protein, both of which are essential for ϕ X174-type primosome assembly, are required for iSDR (Masai and Arai, 1988b). In the present study, we have measured both iSDR and cSDR in mutant cells in which ϕ X174-type primosome assembly is completely prevented by a *priA* mutation without loss of viability. We have discovered that both iSDR and cSDR strictly require the function of PriA protein. These results strongly support our proposal that the ϕ X174-type primosome constitutes replication forks during the course of iSDR and cSDR.

Results

priA1::kan mutants are broth sensitive

When the *priA1::kan* allele was introduced from the original mutant strain (EL500) into several strains of *E. coli*, including AB1157, AQ634 and the parental strain (DPB271) from which EL500 was derived (Lee and Kornberg, 1991), the resultant cells unexpectedly exhibited sensitivity to broths such as LB (Figure 1). Typical plating efficiencies of *priA1::kan* cells were 10^{-3} to 10^{-4} on LB plates and 10^{-1} on minimal plates. Revertants often appeared which showed various degrees of resistance to the broth (see below). Since the original *priA1::kan* strain was constructed on LB plates, the strain is most likely to contain a suppressor mutation(s) which allows the cells to grow in LB broth. One exception was strain MC1061, *priA1* derivatives of which displayed only a slight degree of broth sensitivity (Figure 1).

The *priA1::kan* mutation blocks iSDR

When the *priA1::kan* allele was transduced into AQ634, a thymine-requiring strain, the resultant transductant (AQ8851) grew very slowly in minimal medium. The parental AQ634, after 60 min of thymine starvation, continued DNA synthesis for several hours, despite the presence of Rif and Cam (iSDR; Figure 2A). On the other hand, AQ8851 (*priA1::kan*) exhibited no persistent DNA synthesis after a period (190 min) of thymine starvation.

Fig. 1. Broth sensitivity of *priA1::kan* mutants. About 10^5 cells of cultures grown overnight in M9G were spread on M9G, M9GCAA or LB plates. The plates were incubated at 37°C for 46 h. (A–C) Three clones of AQ7679 (*priA1::kan* transductants of DPB271); (D–F) three clones of AQ7677 (*priA1::kan* transductants of MC1061).

When pLE042 carrying the wild-type *priA* gene was introduced into AQ8851, iSDR was restored to a level comparable with that of the wild-type. Introduction of pHM7182, a pBR322 derivative which carries an A site in place of the *n'-pas* (Masai *et al.*, 1990) and is able to replicate in the *priA* mutant cells (see below), did not restore the incorporation (Figure 2B), showing that the observed incorporation is due to chromosomal DNA synthesis, not replication of the introduced plasmid. These results clearly demonstrate that a function of PriA protein is essential for the process of iSDR.

PriA-dependent primosome assembly is required for initiation of iSDR at *oriM*

The *priA1* mutant is not only defective in SDR, but also deficient in P1 transduction and Hfr conjugation (T.Kogoma, G.Cadwell and T.Asai, unpublished results), suggesting that *priA* is required for homologous recombination as well. Since iSDR also requires homologous recombination functions, most likely to generate a D-loop (Asai *et al.*, 1993), it was possible that PriA was required for D-loop formation rather than for primosome assembly in iSDR. Therefore, we first isolated from the *priA1::kan* mutant a broth-resistant revertant, *spa-47*, that partially recovered recombination proficiency but was still defective in iSDR (data not shown).

We have recently demonstrated a novel DNA replication that is triggered by a double-strand break artificially introduced at a *cos* site on a plasmid by the λ terminase. The replication, termed RDR (for homologous recombination-dependent DNA replication), completely depends on homologous recombination functions and can occur in the presence of Rif and Cam, resembling iSDR (Asai *et al.*, 1994b). In contrast to iSDR, however, RDR can be manifested in cells not induced for the SOS response if

Fig. 2. iSDR in *priA1::kan* strains. (A) AQ634 was grown in M9G to the exponential phase (2×10^8 cells/ml). Half of the culture was starved of thymine for 60 min to induce SOS responses and the other was untreated (Magee and Kogoma, 1990). (B) AQ8851 (*priA1::kan*) without plasmid (circles), AQ8851/pEL042 (squares) or AQ8851/pHM7182 (triangles) were either untreated or starved of thymine for 190, 120 or 210 min respectively. The doubling times of AQ634 and AQ8851 were 50 min and 100 min respectively in M9G. DNA synthesis in the presence of Cam (150 μ g/ml) and Rif (200 μ g/ml) was measured as described previously (Magee and Kogoma, 1990). Not shown in the figure are the values of DNA synthesis from uninduced cultures of AQ8851 and AQ8851/pHM7182, which were almost identical to those of the respective induced cultures. Filled symbols, induced for SOS responses; open symbols, uninduced.

the plasmid also carries a chi (χ) site. The χ site is an 8 bp sequence that is recognized by RecBCD enzyme (exonuclease V) and attenuates the nuclease activity of the enzyme (Kowalczykowski *et al.*, 1994). The strains used in the experiment shown in Figure 3 harbored two plasmids, pHK χ 1-*cos* and pFM123. pFM123 carries the genes for λ terminase, the expression of which is under the cI857 temperature-sensitive repressor. During growth at 30°C, a small amount of terminase was expected to be produced from the plasmid and to introduce double-strand breaks at the *cos* site on some of the pHK χ 1-*cos* molecules. The ends of the linearized pHK χ 1-*cos* molecules would then invade intact molecules by the actions of RecBC(D) and RecA, leading to D-loop formation (Asai *et al.*, 1994b). As shown in Figure 3A, the copy number of pHK χ 1-*cos* in uninduced wild-type cells increased about 5-fold during a 5 h incubation in the presence of Rif and Cam. Similarly, the same plasmid in *priA1::kan spa-47* double mutant cells also continued to replicate under identical conditions, although the rate was slightly slower than in the wild-type during the first 3 h (Figure 3A). The result indicated that the *priA1::kan spa-47* double mutant is proficient in RDR. We inferred from the result that the double mutant is capable of D-loop formation, since initiation of RDR depends on D-loop formation (Asai *et al.*, 1994b).

One of the origins of iSDR, designated *oriM1*, overlaps with *oriC* (Magee *et al.*, 1992; Asai *et al.*, 1994a). Replication of the *oriM1* region after thymine starvation was measured in wild-type and *priA1::kan spa-47* cells. The copy number increase in *oriM1* after an inducing treatment was greatly diminished in AQ9247 (*priA1::kan spa-47*) compared with that in the wild-type (Figure 3B). Presumably, a PriA-independent priming activated by the *spa-47* mutation could prime replication at the D-loops formed after artificial double-strand breaks, i.e. RDR, but not initiation at *oriM1*. These results strongly suggest that *oriM1*-dependent initiation of iSDR requires the PriA function specifically for priming of DNA replication at *oriMs*.

Fig. 3. Relative copy number increase of ccc molecules of pHK χ 1-*cos* and the *oriM1* region on the chromosome in wild-type and *priA1::kan spa-47* mutant cells. AQ634 (wild-type, open symbols) and AQ9247 (*priA1::kan spa-47*, filled symbols) carrying pHK χ 1-*cos* and pFM123 were grown at 30°C to 2×10^8 cells/ml in M9GCAA medium containing [³H]thymine (2 μ Ci/8 μ g/ml). (A) The copy number increase in the pHK χ 1-*cos* plasmid in uninduced AQ634 and AQ9247 was determined as described in Materials and methods. (B) The copy number of the chromosomal *oriM1* region in thymine-starved cells was determined as described in Materials and methods. AQ634 and AQ9247 carrying pHK χ 1-*cos* and pFM123 were starved for thymine for 80 min and 120 min respectively to induce a similar level of SOS response. The level of SOS induction during thymine starvation was monitored by measuring the β -galactosidase activity expressed from an *sfiA::lacZ*⁺ operon fusion carried by lysogenized cI857(Ind⁻) phage. These treatments gave ~300 Miller units of β -galactosidase.

PriA is essential for cSDR

AQ666 (*rnhA224*) displayed continuous incorporation of [³H]thymine in the presence of Cam, which represents cSDR (Figure 4). Introduction of *priA1::kan* into AQ666 generated AQ8853, which grew very slowly and was extremely sensitive to the broth. Incorporation of [³H]thymine leveled off within 1 h after addition of Cam in AQ8853 (*rnhA224 priA1::kan*). cSDR was restored to the original level in AQ8853 when pLE042 expressing the wild-type PriA protein was introduced (Figure 4). These results demonstrate that PriA protein is also essential for the process of cSDR.

Fig. 4. cSDR in *rhA*224 *priA*::*kan* strains. Cells were grown in M9G to exponential phase ($2\text{--}3 \times 10^8$ cells/ml) and DNA synthesis in the presence of chloramphenicol was measured as described previously (Torrey and Kogoma, 1987). Open circles, AQ634 (*rhA*⁺); filled circles, AQ666 (*rhA*224); open squares, AQ8853 (*rhA*224 *priA*::*kan*); filled squares, AQ8853 carrying pLE042.

Inactivation of *priA* is lethal to cells that depend on cSDR for viability

When a mutation in *rhA* activates cSDR, *E. coli* cells can grow in the absence of DnaA protein or the *oriC* site (Kogoma and von Meyenburg, 1983). To examine whether *priA*⁺ is essential for the viability of those cells in which cSDR is the only means to replicate the chromosome, P1 transduction of the *priA*::*kan* mutation into AQ3519 (*rhA*::*cat dnaA*::*Tn10*) was attempted. This strain can tolerate the absence of DnaA function, since the cSDR pathway is activated by the *rhA* mutation. No transductants were obtained from this strain, whereas more than 300 transductants were obtained from AQ3764 (*rhA*::*cat dnaA*⁺) under the same conditions (data not shown). The result suggests that inactivation of *priA* in AQ3519 is lethal to the cell.

AQ8101 (*rhA*::*cat dnaA*46) could grow at 42°C owing to cSDR, despite the *dnaA*(Ts) mutation (Figure 5C and G). The *priA* mutation was introduced into the strain at 30°C and the transductants were tested for temperature sensitivity. The transductant AQ9875 was temperature-sensitive for growth (Figure 5A and B). Both AQ8851 (*priA*::*kan*) and AQ9874 (*rhA*::*cat priA*::*kan*) grew at 42°C (Figure 5E, F and H). The growth of the *priA* mutants (AQ8851 and AQ9874) at 30°C was extremely slow (Figure 5E, F and H). These results indicated that PriA protein is essential for viability when the DnaA/*oriC*-dependent replication pathway is inactivated and duplication of the chromosome solely depends on cSDR.

The *priA*::*kan* mutation does not inhibit DnaA-dependent plasmid replication

The above results suggest that chromosome replication can be primed either by the DnaA-dependent primosome (from *oriC*) or by the PriA-dependent ϕ X174-type primosome (from D-loops and R-loops). To determine which primosome drives the replication of plasmid replicons that reside in *E. coli*, we examined the transformation efficiency of various plasmids into the *priA*::*kan* strain (Table I). AQ7677 (a *priA*::*kan* derivative of MC1061), which is broth-resistant (Figure 1), was used and transformants

Fig. 5. Lethal effect of *priA*::*kan* on an *rhA dnaA* double mutant. Cells of various strains were spread on M9G plates and the plates were incubated either at 30°C (right) or at 42°C (left) for 3 days. (A, B) Two clones of AQ9875 (*rhA*::*cat dnaA*46 *priA*::*kan*); (C, G) two clones of AQ8101 (*rhA*::*cat dnaA*46); (D) AQ3764 (*rhA*::*cat*); (E, F) two clones of AQ9874 (*rhA*::*cat priA*::*kan*); (H) AQ8851 (*priA*::*kan*).

were selected on LB containing appropriate antibiotics. As previously reported (Lee and Kornberg, 1991; Zavitz and Mariani, 1992), the DnaA-independent pBR322 plasmid (derived from pMB1) gave no transformants in this strain (relative transformation frequency less than 8.3×10^{-10}), whereas pEL042, which carries a wild-type *priA* gene, gave a relative frequency of $\sim 0.5 \times 10^{-2}$. RSF1030 (Crosa *et al.*, 1975), another class of DnaA-independent plasmid belonging to a different incompatibility group, also gave no transformants in *priA*::*kan*. On the other hand, all other DnaA-dependent replicons, such as pSC101 (Hasunuma and Sekiguchi, 1977), F (Hansen and Yarmolinski, 1986), R6K (Lovett *et al.*, 1972; Kline, 1988), Rts1 (Kamio *et al.*, 1984), RK2 (Gaylo *et al.*, 1987) and *oriC* (Yamaguchi *et al.*, 1982) plasmids, gave relative transformation frequencies ranging from 2.3×10^{-3} to 1.2×10^{-2} (Table I). Not only the mini-F plasmid (pKU102 carrying a 9 kb *EcoRI* fragment containing both *ori-1* and *ori-2*) but also a plasmid carrying only *ori-2* (pKP1013; Maki *et al.*, 1983) could transform into *priA*::*kan*, indicating that RepE/DnaA-dependent replication from *ori-2* does not require PriA. The previous report that the *oriC* plasmid poorly transforms *priA* null mutant cells is due probably to poor viability on LB plates of the *priA*::*kan* strain which was used in that study (Lee and Kornberg, 1991). A *priA*::*kan* derivative of DPB271, similar to EL500 used in the previous report, indeed did not grow well on LB plates (Figure 1). In contrast, MC1061 *priA*::*kan* used in the present study grew well on LB, due, presumably, to a pre-existing suppressor

Table I. Transformation frequencies of *priA*⁺ and *priA1::kan* strains with various plasmids

Plasmid	Replicon	Drug resistance used for selection	Dependence on DnaA	Transformation frequency (per µg)		Relative frequency
				AQ7677 (<i>priA1::kan</i>)	AQ7676 (<i>priA</i> ⁺)	
pBR322	pMB1	Amp	—	<10 ³	1.2×10 ⁹	<8.3×10 ⁻¹⁰
pEL042 (with <i>PriA</i>)	pUC	Amp	—	3.3×10 ⁶	6.8×10 ⁸	4.9×10 ⁻³
pHM7182 (with A site)	pMB1	Amp	ND	3.4×10 ⁶	2.0×10 ⁸	1.7×10 ⁻²
RSF1030	RSF1030	Amp	—	<10 ³	2.6×10 ⁸	<3.9×10 ⁻⁹
pSC101	pSC101	Tet	+	6.7×10 ⁵	7.7×10 ⁷	8.7×10 ⁻³
pKU102	mini-F	Amp	+	9.4×10 ⁵	1.6×10 ⁸	6.1×10 ⁻³
pKP1013	mini-F (with only <i>ori-2</i>)	Cam	+	1.7×10 ⁶	1.8×10 ⁸	9.5×10 ⁻³
R6K	R6K	Amp	+	1.7×10 ⁵	1.4×10 ⁷	1.2×10 ⁻²
pKU101	mini-Rts1	Amp	+	3.8×10 ⁶	6.3×10 ⁸	6.0×10 ⁻³
pRK2501	RK2	Tet	+	5.9×10 ⁵	2.6×10 ⁸	2.3×10 ⁻³
pKY131	oriC	Amp	+	6.7×10 ⁶	5.8×10 ⁸	1.2×10 ⁻²

ND, not determined.

Amp, ampicillin; Tet, tetracycline.

Numbers of transformants were counted after incubation of plates at 37°C for 24 h.

mutation in the parental strain MC1061 (Figure 1). Although the replication of ColE1-type plasmids (pBR322 and RSF1030) strictly requires *PriA*, replacement of the *n'*-*pas* of pBR322 with an A site (pHM7182; Masai *et al.*, 1990) caused the replication to become *PriA*-independent (Table I), showing that the DnaA-dependent primosome, if appropriately assembled, can functionally replace the *PriA*-dependent one in the replication of ColE1-type plasmids *in vivo*.

These results show that each plasmid replicon requires either DnaA or *PriA* for replication. Thus, these bacterial replicons can be classified into two groups; namely DnaA-dependent and *PriA*-dependent.

Discussion

In *E. coli*, at least two types of distinct primosomes have been identified *in vitro*. One that is assembled at *oriC* is dependent on DnaA protein (the *oriC* primosome; Kornberg and Baker, 1991). A DnaA-dependent primosome can also be assembled at the A site, a single-stranded stem-loop structure recognized by DnaA protein (the ABC-primosome; Masai *et al.*, 1990). The other primosome depends on *PriA* protein and is assembled at *n'*-*pas* (the ϕ X174-type primosome; Allen and Kornberg, 1993). Although the ϕ X174-type primosome drives replication forks during the course of ColE1-type plasmid replication (Minden and Marians, 1985; Masai and Arai, 1988a) and fragments containing *n'*-*pas* have been isolated from a variety of plasmid replicons (Zipursky and Marians, 1981; Nomura *et al.*, 1982; Imber *et al.*, 1983; Bahk *et al.*, 1988; Nomura *et al.*, 1991), the involvement of this primosome in chromosomal replication has not been conclusively shown. In fact, the following lines of evidence seem to preclude any essential role of the ϕ X174-type primosome in the normal chromosome replication process. (i) The entire cycle of *oriC* plasmid replication can be reconstituted *in vitro* from a set of purified proteins that does not include *PriA* protein (Funnel *et al.*, 1986). (ii) The crude extract prepared from the *priA* null strain

can support the wild-type level of *dnaA*-dependent *oriC* plasmid replication (E.H.Lee, unpublished data). (iii) Inactivation of the *priA* gene does not affect cell viability (Lee and Kornberg, 1991; Nurse *et al.*, 1991).

In this study, we have demonstrated that *PriA* protein is absolutely essential for iSDR as well as for cSDR, both of which can occur in the complete absence of DnaA protein (Kogoma and von Meyenburg, 1983; Magee *et al.*, 1992). Furthermore, the viability of cells whose chromosome replication depends on cSDR was lost upon inactivation of *priA*. At least for iSDR, *PriA* appears to be involved in initiation at a step after D-loop formation, since the *priA1::kan spa-47* double mutant is defective in iSDR although RDR can be manifested in the mutant. The double mutant is proficient in SOS responses and therefore activation and amplification of the *recA* gene product, which are necessary and sufficient for induction of iSDR, should occur in this mutant strain. We wish to propose that *PriA* is needed for priming iSDR. This proposal is supported by our earlier finding that DnaT (protein i) and DnaC, essential for the assembly of the ϕ X174-type primosome, are required for iSDR (Masai and Arai, 1988b). The isolation of a *dnaB* mutant which appears to be defective specifically in iSDR also lends support to the proposal (Kogoma, 1976). Our finding strongly suggests that the ϕ X174-type primosome plays a crucial role in DnaA/*oriC*-independent replication of the *E. coli* chromosome. A possible role of the ϕ X174-type primosome in a back-up system for *E. coli* chromosome replication has also been previously suggested (Seufert and Messer, 1986).

How could *PriA* protein trigger primosome assembly in iSDR and cSDR? One possible mechanism is assembly of the ϕ X174-type primosome on displaced single-stranded DNA within the D-loop or the R-loop. In replication of ColE1-type plasmids in *rmhA*⁺ cells, either the ϕ X174-type or the ABC-type primosome can be assembled at a *pas* (*n'*-*pas* or A site, respectively) that is present on the displaced single-stranded DNA after leading strand synthesis by DNA polymerase I (Minden and Marians,

Fig. 6. Functions of two distinct types of primosomes in replication of bacterial replicons. The *E. coli* chromosome or various plasmid replicons replicate in a manner dependent on either of the two types of primosomes, DnaA-dependent or PriA-dependent. Under normal growing conditions, the DnaA-dependent primosome is responsible for host chromosomal replication from *oriC*. However, the PriA-dependent primosome plays a major role in iSDR or cSDR, which does not require *oriC* or DnaA but requires RecA. In *oriC*-dependent replication, strand opening by DnaA protein triggers the reaction, whereas D-loops or R-loops play crucial roles in initiation of iSDR or cSDR, respectively. The origins for iSDR and cSDR are called *oriMs* and *oriKs*, respectively. See text for details.

1985; Masai *et al.*, 1990). In iSDR, the ϕ X174-type primosome may be similarly assembled on displaced single-stranded DNA in a D-loop generated by recombination processes (Asai *et al.*, 1993). In a very similar manner, R-loops on the chromosome could also make *n'*-*pas* on the displaced single-stranded DNA available for primosome assembly. This may be analogous to the extensive RNA–DNA hybrid formation downstream of the origin of pBR322 (nearly 3 kb on a 4.4 kb template) *in vitro*, which generates displaced single-stranded DNA containing an *n'*-*pas* (Parada and Marians, 1989).

This model demands that the *E. coli* chromosome contains one or more *n'*-*pas*. Contrary to expectation, however, attempts to identify *n'*-*pas* on the *E. coli* chromosome have so far been unsuccessful (N.Nomura, unpublished results). Furthermore, *n'*-*pas* is not present within or near *oriM1* (overlapping with the *oriC* site; Stuitje *et al.*, 1984; Asai *et al.*, 1994a; N.Nomura, unpublished results). In replication of ColE1-type plasmids, *n'*-*pas* on the lagging strand template is not essential, although its absence results in inefficient lagging strand synthesis *in vitro* and reduced copy number *in vivo* (Van der Ende *et al.*, 1983; Masai and Arai, 1988a). On the other hand, PriA protein is absolutely required for their replication, regardless of the presence or absence of *n'*-*pas*. This suggests that PriA protein contributes to the primosome assembly even in the absence of *n'*-*pas*. *In vitro*, the dATPase activity of the purified PriA protein can be weakly activated in the absence of SSB by non-specific single-stranded DNA, such as M13 SS, which does not contain *n'*-*pas* (Shlomai and Kornberg, 1980a,b). It was also reported that PriA protein could form a complex with SSB-coated M13 SS *in vitro*, possibly through interaction with SSB (Allen and Kornberg, 1993). These considerations raise the possibility that PriA protein interacts with single-stranded DNA in a manner independent of *n'*-*pas* and suggest that a primosome can be assembled on single-stranded DNA without a canonical *n'*-*pas*, albeit with reduced efficiency.

The ability of *rnhA* mutants to tolerate inactivation of

DnaA clearly indicates that the PriA-dependent primosome can sustain replication of the entire chromosome in the absence of DnaA function. An examination of various plasmid replicons for their ability to replicate in the *priA1::kan* strain has indicated that PriA protein is essential for plasmids such as ColE1-type plasmids that do not require DnaA protein, and that DnaA protein-dependent replicons, including pSC101, F, R6K, Rts1, RK2 and *oriC* plasmids, do not require PriA protein (Table I). Interestingly, replication of a pBR322 derivative became independent of PriA upon introduction of an A site, on which the DnaA-dependent ABC primosome can be assembled (Masai *et al.*, 1990). This plasmid is presumably replicated in a manner dependent on DnaA, although this has not been examined. DnaA-dependent replication *in vitro* of pBR322 was also previously reported (Seufert and Messer, 1987; Parada and Marians, 1991), although the mechanism of priming in these cases may be different from that by the ABC primosome on an A site. We propose that each bacterial replicon primarily relies on either one of the two functionally equivalent but distinct primosomes that *E. coli* possesses; one that depends on DnaA protein and the other on PriA protein (Figure 6).

The replication origins of DnaA-dependent replicons, including the plasmids studied in this work (Table I), contain one or more DnaA boxes (Bramhill and Kornberg, 1988). These DnaA boxes are additional to a specific recognition sequence for each cognate initiator protein. Our results lend strong support to the speculation that the DnaA protein–DnaA box interaction in many of these replicons is necessary to achieve DnaB entry to the site for subsequent primosome assembly (Bramhill and Kornberg, 1988; Skarstad and Boye, 1994).

Evolutionarily, the DnaA–*oriC* system is well conserved in many eubacteria examined thus far (Yoshikawa and Ogasawara, 1991; Ogasawara and Yoshikawa, 1992). However, no DnaA homologue has yet been found in eukaryotic species. The PriA–R-loop or PriA–D-loop system has been identified only in *E. coli*, although a

Table II. *E. coli* strains

Strain	Relevant genotype	Reference/construction
AQ634 ^a	<i>thyA rnhA</i> ⁺	Ogawa <i>et al.</i> , 1984
AQ666 ^a	<i>thyA rnhA224</i>	Ogawa <i>et al.</i> , 1984
AQ705 ^a	<i>thyA dnaA46</i>	Laboratory collection
AQ3519 ^a	<i>thyA rnhA339::cat dnaA850::Tn10</i>	Kline <i>et al.</i> , 1986
AQ3764 ^a	<i>thyA rnhA339::cat</i>	Laboratory collection
AQ7676 ^b	<i>priA</i> ⁺	as MC1061 (Meissner <i>et al.</i> , 1987)
AQ7677 ^b	<i>priA1::kan</i>	AQ7676×P1.AQ7679, select Km ^r
AQ7678	<i>recD1903::mini-tet</i>	as DPB271 (Biek and Cohen, 1986)
AQ7679	<i>recD1903::mini-tet priA1::kan</i>	as EL500 (Lee and Kornberg, 1991)
AQ8101 ^a	<i>thyA dnaA46 rnhA339::cat</i>	AQ705×P1.AQ3519, select Cm ^r
AQ8845 ^b	<i>priA1::kan /F'134</i>	AQ7677×CGSC4264, select Leu ⁺ His ⁺
AQ8851 ^a	<i>thyA rnhA</i> ⁺ <i>priA1::kan</i>	AQ634×P1.AQ8845, select Km ^r
AQ8853 ^a	<i>thyA rnhA224 priA1::kan</i>	AQ666×P1.AQ8845, select Km ^r
AQ9247 ^a	<i>thyA rnhA</i> ⁺ <i>priA1::kan spa-47</i>	AQ8851, spontaneous broth-resistant
AQ9874 ^a	<i>thyA rnhA339::cat priA1::kan</i>	AQ3764×P1.AQ8845, select Km ^r
AQ9875 ^a	<i>thyA rnhA339::cat dnaA46 priA1::kan</i>	AQ8101×P1.AQ8845, select Km ^r
CGSC4264 ^c	F'134 (<i>priA</i> ⁺ <i>leu</i> ⁺) <i>metB</i>	Low, 1973

The remaining genotypes are: ^aF⁻ *his-29 trpA9605 pro ilv metB deoB* (or C); ^bF⁻ *araD Δ(araABC-leu)7679 ΔlacX74 galU galK rpsL thi*; ^c*argG6 metB his-1 leu-6 ml xyl-7 malA gal-6 lacY tonA2 str-104*.

homologue of PriA has been found in the photosynthetic bacterium *Rhodospirillum rubrum* (Lee *et al.*, 1990). Initiation of replication from R-loops is much less efficient than from *oriC* (von Meyenburg *et al.*, 1987), but the former may be more promiscuous, due to its low specificity. Opportunistic persistence of a transcript in association with the template DNA could activate replication. cSDR is known to occur at multiple sites (*oriKs*) on the chromosome (Kogoma, 1986). In primitive cells ancestral to the present-day bacteria, this kind of less specific mode of initiation, rather than the highly specific DnaA–*oriC* system, is likely to have been utilized. The latter may have evolved to meet the demand for replicating the genome at a maximum rate in a controlled manner. Appearance of diverse replicons may also have imposed the need for each replicon to be maintained without being interfered with by others. Thus, each replicon is under the control of a specific protein, an initiator Rep protein. It is an interesting question how the PriA system, which probably outdated the divergence of bacteria and eukaryotes, has been conserved in the replication of present-day eukaryotic cells. Identification of PriA homologues in eukaryotes, if any, will be one approach to answer this question.

Materials and methods

Media and growth conditions

Unless otherwise stated, cells were grown at 37°C with aeration by shaking in M9 salts–glucose medium supplemented with thymine (8 µg/ml), thiamine hydrochloride (2 µg/ml) and required amino acids (50 µg/ml) (M9G) or casamino acids (0.2%; Difco Laboratories, Detroit, MI) (M9GCAA). Luria broth (LB) has been described previously (Miller, 1992). LBGT is LB supplemented with glucose (0.1%) and thymine (8 µg/ml). For selection of antibiotic-resistant cells, antibiotics were added at the following concentrations: ampicillin (40 µg/ml), kanamycin (25 µg/ml), tetracycline (10 µg/ml) and chloramphenicol (25 µg/ml).

Reagents and isotopes

Reagents were purchased from Sigma Chemical Co. (St Louis, MO), Wako Pure Chemical Industries Ltd (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan). [Methyl-³H]thymine and [α-³²P]deoxycytidine-5'-triphosphate were from New England Nuclear Corp. (Boston, MA).

E. coli strains and plasmids

The *E. coli* strains used in this study are listed in Table II. Two *priA1::kan* alleles have been independently constructed, either by replacing a part of the *priA* coding sequence with a *kan* gene or by simply inserting a *kan* gene fragment. We propose to designate these *priA1::kan* (Lee and Kornberg, 1991) and *priA2::kan* (Nurse *et al.*, 1991). In this study, strains were constructed by phage P1-mediated transduction (Lennox 1955). Since P1 phage grew poorly on *priA1::kan* strains, P1 lysates were made from strains into which a F'134 episome or ColE1-based plasmid carrying a wild-type *priA* was introduced. pEL042 is based on pTZ18R (purchased from Pharmacia) and carries a *priA*⁺ gene (Lee *et al.*, 1990). pHM7182 is a derivative of pBR322 in which the n' site on the lagging strand template is replaced by an A site (identical to pHM7203 described in Masai *et al.*, 1990). pHKχ1-*cos* (Asai *et al.*, 1994b) and pMF123 (Murialdo, 1988) were previously described. The R6K (Lovett *et al.*, 1972) and mini-Rts1 (Kamio *et al.*, 1984) plasmids were generous gifts from Dr Inuzuka (Fukui Medical School) and Dr Terawaki (Shinshu University School of Medicine), respectively. pKU101 was constructed by joining the 1.86 kb *EcoRI*–*HindIII* fragment containing the mini-Rts1 genome (Kamio *et al.*, 1984) with the 1.1 kb *EcoRI*–*HindIII* fragment containing β-lactamase (derived from pHM6050; Masai and Arai, 1989b). pKU102 was constructed by joining the 9 kb mini-F *EcoRI* fragment from pML31 (Lovett and Helinski, 1976) with the same 1 kb β-lactamase DNA fragment whose ends had both been converted to *EcoRI* sites. pKY131 (Yamaguchi *et al.*, 1982) and pKP1013 (Maki *et al.*, 1983) were provided by Dr Yamaguchi (Kanazawa University) and Dr Wada (Kyoto University Virus Institute), respectively. pSC101 (Cohen and Chang, 1977), RSF1030 (Crosa *et al.*, 1975) and pRK2501 (mini-RK2 plasmid; Figurski and Helinski, 1979) were laboratory stocks.

Isolation of the *spa-47* mutant

AQ8851 grown in M9GCAA was diluted and approximately 10⁵ cells were plated onto LBGT. After incubation at 37°C, fast growing colonies were picked and were further characterized. One of these broth-resistant revertants carrying extragenic suppressors of the broth sensitivity of the *priA1::kan* mutant was found to regain the ability to replicate pBR322, to become resistant to UV and mitomycin C and to be partially proficient in homologous recombination. It was designated *spa-47* for suppressor of *priA*. The details of characterization of the *spa-47* mutation will be described elsewhere.

Measurement of iSDR and cSDR

The measurement of iSDR after thymine starvation and of cSDR in the presence of chloramphenicol was conducted as described previously (Magee and Kogoma, 1990; Torrey and Kogoma, 1987). Strains harboring a plasmid were grown in the presence of ampicillin.

Transformation with plasmid DNA

Transformation was conducted by electroporation using an electroporator (Invitrogen Corp., San Diego, CA) or Gene Pulser (Bio-Rad, Hercules,

CA). For regular transformation, 100 ng of plasmid DNA was mixed with 40 µl of washed and concentrated cells and all or an appropriate portion of the cell suspensions were plated on M9GTT plates containing ampicillin. To measure transformation efficiency with various plasmids, 40 µl of washed and concentrated MC1061 or MC1061 *priA1::kan* cells were mixed with 1–2 ng of plasmid DNA and an appropriate portion of the suspension [20–100 µl for AQ7677 (*priA1::kan*) and 100 µl of a 1/100 dilution of AQ7676 (*priA⁺*)] was plated on LB plates containing appropriate antibiotics after addition of 1 ml of SOC (Sambrook *et al.*, 1989) and incubation at 37°C for 1 h. The plates were incubated at 37°C.

Determination of RDR activity

Origin-independent replication of pHK χ 1-*cos* (i.e. RDR) triggered by double-strand breaks was assayed as described before (Asai *et al.*, 1994b). Briefly, cells harboring pHK χ 1-*cos* and pFM123 were grown at 30°C to 2×10^8 cells/ml in M9GCAA containing [3 H]thymine (2 µCi/8 µg/ml). Rif (200 µg/ml) and Cam (150 µg/ml) were added (time 0) and aliquots were taken at intervals. Total DNA was extracted as previously described (Magee *et al.*, 1992; Asai *et al.*, 1993). Amounts of DNA samples normalized to give equal 3 H counts were loaded for agarose gel electrophoresis. After electrophoresis, amounts of plasmid pHK χ 1-*cos* DNA were specifically determined by Southern blot hybridization using a 32 P-labeled probe made from a *kan* fragment of the plasmid (Asai *et al.*, 1994b).

Determination of copy number increase of the *oriM1* region after inducing treatment

Total DNA samples were extracted as previously described (Asai *et al.*, 1993). They were digested with *Bam*HI and loaded on agarose gels (0.8%) for electrophoresis. Southern hybridization was carried out as described using a 32 P-labeled probe prepared with a DNA fragment carrying the *oriC* (*oriM1*) sequence from coordinates 92 to 2191 (Buhk and Messer, 1983).

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