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The ϕX174-type primosome promotes replisome assembly at the site of recombination in bacteriophage Mu transposition

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Initiation of *Escherichia coli* DNA synthesis primed by homologous recombination is believed to require the ϕX174-type primosome, a mobile priming apparatus assembled without the initiator protein DnaA. We show that this primosome plays an essential role in bacteriophage Mu DNA replication by transposition. Upon promoting transfer of Mu ends to target DNA, the Mu transpososome undergoes transition to a prereplisome that permits initiation of DNA synthesis only in the presence of primosome assembly proteins PriA, DnaT, DnaB and DnaC. These assembly proteins promote the engagement of primase and DNA polymerase III holoenzyme, initiating semi-discontinuous replication preferentially at the Mu left end. The results indicate that these proteins play a crucial role in promoting replisome assembly on a recombination intermediate.

Keywords: *in vitro* DNA replication/phage Mu/primosome/replisome/transposition

Introduction

Coupling of DNA synthesis to recombination is an important mechanism involved in DNA repair, genetic exchange and chromosomal replication. Growing evidence suggests interdependence between chromosomal replication and homologous recombination, DNA replication participating in the formation of recombinants and homologous recombination leading to initiation of chromosomal replication (Kogoma *et al.*, 1996). Involvement of the primosome assembly protein PriA in both recombinant formation and recombination-dependent DNA replication in *Escherichia coli* has suggested that it may be part of an apparatus for linking strand exchange with DNA synthesis.

PriA is a constituent of the ϕX174-type primosome, which originally was characterized for its function in converting single-stranded phage ϕX174 DNA to the duplex replicative form (Kornberg and Baker, 1992). It is distinguished from the *oriC*-type primosome by the involvement of host-encoded PriA, PriB, PriC and DnaT proteins in primosome assembly instead of the initiator protein DnaA, which promotes replisome assembly at the bacterial origin of replication. In ϕX174 replication, PriA binds to the unique primosome assembly site (PAS) on single-stranded phage DNA and recruits PriB, PriC and DnaT (Shlomai and Kornberg, 1980; Liu *et al.*, 1996; Ng and Marians, 1996a). With the assistance of the associated matchmaker DnaC, DnaB helicase is then delivered to the complex to form the preprimosome. DnaB within this mobile apparatus interacts transiently with primase to form the primosome (Tougo *et al.*, 1994; Ng and Marians, 1996b), which catalyzes synthesis of RNA primers at many sites on the template to initiate DNA synthesis by the DNA polymerase (pol) III holoenzyme (Ng and Marians, 1996b).

PriA's ability to promote primosome assembly plays an important role in DnaA-independent DNA synthesis such as pBR322 replication (Minden and Marians, 1985). On a preformed replication fork, which is a circular duplex with a single-stranded tail, PriA can promote the assembly of a replisome that catalyzes leading and lagging strand synthesis if a PAS is present on the tail (Wu *et al.*, 1992). However, the ϕX-type primosome is not necessarily required for replication of the bacterial chromosome. DNA replication initiated at *oriC* can be reconstituted *in vitro* without the PriA, PriB, PriC and DnaT proteins (Kaguni and Kornberg, 1984). Strains with *priA* null mutations are viable although they display characteristics of slow growth, filamentous structure, increased sensitivity to DNA-damaging agents and a constantly induced SOS system (Lee and Kornberg, 1991; Nurse *et al.*, 1991). It has been suggested that the ϕX-type primosome may be required for reinitiation should the replisome stall (Nurse *et al.*, 1991). Recent evidence demonstrates that *priA* null strains show poor assimilation of genetic markers by homologous recombination and are defective in DNA double strand break repair (Kogoma *et al.*, 1996). They are also deficient in inducible and constitutive stable DNA replication (iSDR and cSDR) (Masai *et al.*, 1994), forms of chromosomal replication which occur independently of the DnaA protein.

Since iSDR is dependent on homologous recombination functions, a model has been proposed for the function of the ϕX-type primosome in coupling recombination with replication (Asai and Kogoma, 1994; Kogoma, 1996). The potential replication fork is produced when an invading strand displaces one strand of a duplex to form a D-loop structure (Eggleston and West, 1996) and provides the potential primer for leading strand synthesis. The ϕX-type primosome is assembled on the single-stranded region within the D-loop, promoting replisome assembly and establishing a replication fork (Kogoma, 1996). In support of this hypothesis, DnaT and DnaC, which are also involved in the assembly of the ϕX-type primosome, are required for iSDR as well (Masai and Arai, 1988). In addition, PriA can bind to D-loops and related DNA structures (McGlynn *et al.*, 1997). However, the ability of the ϕX-type primosome to promote initiation of replication on a natural recombination intermediate has heretofore not been demonstrated.

Phage Mu DNA replication by transposition resembles the hypothesized mechanisms for DNA replication coupled

Table I. PriA⁻ *Escherichia coli* hosts can support Mu lysogenization but not lytic development

Host strain[a]	Relevant trait	Mu plating efficiency	Frequency of lysogenization
EL501	PriA⁺	1	8×10^{-3}
EL500	PriA⁻	$<10^{-7}$	0.7×10^{-3}
EL502	PriA⁺	0.8	not determined
AT3327	PriA⁺	1	4×10^{-3}
AT3327 *priA1::kan*	PriA⁻	$<10^{-7}$	0.8×10^{-3}

[a]EL501 and EL500 are an isogenic pair; EL500 contains a 1.3 kb insertion in the *priA* gene (*priA1::kan*) (Lee and Kornberg, 1991). EL502 also contains this insertion but has been transformed with plasmid pEL042 expressing PriA (Lee *et al.*, 1990).

to homologous recombination. In Mu transposition, strand exchange is catalyzed by the phage-encoded transposase MuA (for reviews, see Mizuuchi, 1992; Chaconas *et al.*, 1996; Lavoie and Chaconas, 1996). Monomeric MuA binds to specific sequences at each Mu end (Craigie *et al.*, 1984; Kuo *et al.*, 1991), assembling into a tetramer that holds together the two ends (Lavoie *et al.*, 1991). This transpososome introduces a nick at each end, and the resulting 3′-hydroxyl groups are transferred to target DNA (Craigie and Mizuuchi, 1987; Surette *et al.*, 1987; Mizuuchi *et al.*, 1992), producing a branched DNA structure with a potential replication fork at each Mu end.

A specific set of host proteins is required to replicate Mu DNA on this strand transfer intermediate, and MuA plays a key role in controlling access of host proteins to the two potential replication forks (Kruklitis and Nakai, 1994; Nakai and Kruklitis, 1995; Kruklitis *et al.*, 1996). Oligomeric MuA remains tightly bound to both Mu ends in a nucleoprotein complex known as the strand transfer complex (STC1) or type II transpososome (Surette *et al.*, 1987; Lavoie *et al.*, 1991). A group of host factors called Mu replication factors α (MRFα), which includes the molecular chaperone ClpX and at least one additional component ($MRF\alpha_2$) (Kruklitis *et al.*, 1996), removes MuA from STC1 to form a prereplisome, a nucleoprotein complex (STC3) that only allows initiation of Mu DNA synthesis by a specific set of host factors (Nakai and Kruklitis, 1995). These factors include replication proteins such as DnaB, DnaC and DNA pol III holoenzyme, which are known to be required for Mu DNA synthesis *in vivo*, and a group of host factors called MRFβ, previously used in the reconstituted system in partially purified form.

In this study, we identify the host factors in MRFβ as PriA, PriB and DnaT. We characterize the function of these proteins in promoting Mu replication on the Mu strand transfer intermediate.

Results

Mu replication by transposition in vivo is dependent on the priA gene function

We examined the ability of Mu to grow in *E.coli* strains with inactivating mutations in the *priA* gene. Two *E.coli* strains with *priA* null mutations (PriA⁻) supported Mu lysogenization but were unable to support lytic growth (Table I). The ability to support Mu lytic growth was restored by transformation with a plasmid expressing PriA (Table I).

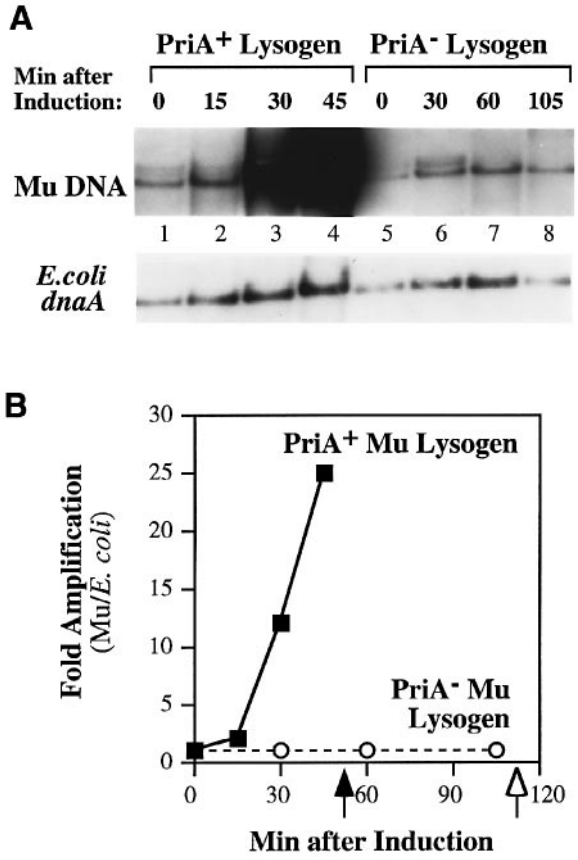

Fig. 1. Requirement for the *priA* function in bacteriophage Mu DNA replication *in vivo*. (**A**) Southern blot of DNA prepared from induced cultures of Mu lysogens AT3978 (PriA⁺) and AT3978 *priA1::kan* (PriA⁻) probed with Mu-specific and *E.coli dnaA*-specific sequences. (**B**) Quantitation of Mu DNA amplification relative to an *E. coli*-specific marker (*dnaA*). Solid and open arrows indicate the time at which lysis occurred for the PriA⁺ and PriA⁻ Mu lysogens, respectively.

To determine whether this block in lytic development specifically affected Mu replication by transposition, we examined amplification of Mu DNA in induced PriA⁺ and PriA⁻ Mu lysogens (*his*::Mucts62). Both lysogens eventually lysed after heat induction and, as expected, the PriA⁺ lysate was highly infectious [$>10^{10}$ plaque-forming units (p.f.u.) per ml] whereas the PriA⁻ lysate had no detectable titer ($<10^3$ p.f.u. per ml). Southern blot analysis of DNA isolated from the induced PriA⁺ Mu lysogen (Figure 1A, lanes 1–4) indicated that Mu DNA was amplified at least 25-fold relative to a host-specific marker (*dnaA*) before lysis (Figure 1B). No amplification was detected in the induced PriA⁻ lysogen (Figure 1A, lanes 5–8, and Figure 1B) even though reconstruction experiments indicated that as little as a 2-fold increase in Mu DNA could be detected using this Southern blot technique (data not shown). These results indicate that Mu was unable to undergo even one round of replication by transposition *in vivo* in the absence of PriA.

PriA and additional ϕX-type primosome constituents are required for Mu DNA replication in vitro

In the *in vitro* transposition system, STC1 is formed using a supercoiled plasmid bearing a mini-Mu element as donor

substrate and a second plasmid as target (Mizuuchi, 1983). Mu DNA in STC1 can be replicated to form a cointegrate using a reconstituted system composed of an eight-protein system [DnaB, DnaC, primase, DNA pol III holoenzyme, DNA pol I, DNA gyrase, single-strand binding protein (SSB) and DNA ligase] supplemented with MRFα (or ClpX and MRFα_2) and MRFβ (Kruklitis and Nakai, 1994; Nakai and Kruklitis, 1995). MRFα and MRFβ can be supplied separately (each as fraction III) or together in a crude enzyme fraction (fraction II). We determined whether PriA was an essential component of this system.

The eight-protein system supplemented with fraction II from a PriA$^-$ *E.coli* strain did not support Mu DNA replication (Figure 2A). The addition of purified PriA restored only low levels of replication activity, while the addition of both PriA and DnaT restored activity to that obtained with fraction II from a wild-type strain, suggesting that Mu DNA replication was dependent on both PriA and DnaT and that our PriA$^-$ fraction II was also deficient in DnaT activity. Using a reconstituted assay for the replication of ϕX174 single-stranded DNA, we found that our PriA$^-$ fraction II was indeed partially deficient in DnaT activity relative to a fraction II from a PriA$^+$ strain (data not shown).

PriA was a necessary component of MRFβ which provides complementing activity in the reconstituted Mu replication system. While an MRFα fraction III prepared from a PriA$^-$ strain had complementing activity comparable with MRFα from a PriA$^+$ strain (data not shown), the MRFβ fraction III prepared from a PriA$^-$ strain showed only background levels of activity (Figure 2B). Unlike the PriA$^-$ fraction II, full activity was restored to MRFβ (PriA$^-$ fraction III) by the addition of purified PriA alone (Figure 2B). The specific activity of MRFβ is increased 10- to 15-fold during preparation of fraction III, and therefore the enrichment of low levels of DnaT in fraction II as well as removal of unwanted proteins most likely yielded a MRFβ(PriA$^-$) fraction with sufficient DnaT activity to promote high levels of Mu DNA replication.

MRFβ could be replaced by purified PriA, PriB and DnaT (Figure 2C). Cointegrate production was absolutely dependent on PriA, DnaBC and MRFα as well as the ϕX components PriB and DnaT (Table II). The small amounts of cointegrate production apparent when either PriB or DnaT was omitted individually are most likely due to low levels of PriB and DnaT in the MRFα fraction, detected using the reconstituted ϕX174 replication assay (data not shown). The lack of any replication when both are omitted (Table II) strongly supports the conclusion that PriA is not acting independently of PriB and DnaT during Mu DNA replication but is assembling a multi-component primosome like the one characterized in ϕX174 replication. We could not determine the dependence of Mu replication on PriC because high levels of PriC activity were present in the MRFα fraction (data not shown). MRFα cannot be replaced with purified PriC and ClpX (Table II), indicating that at least one additional factor besides these two proteins is an essential MRFα component.

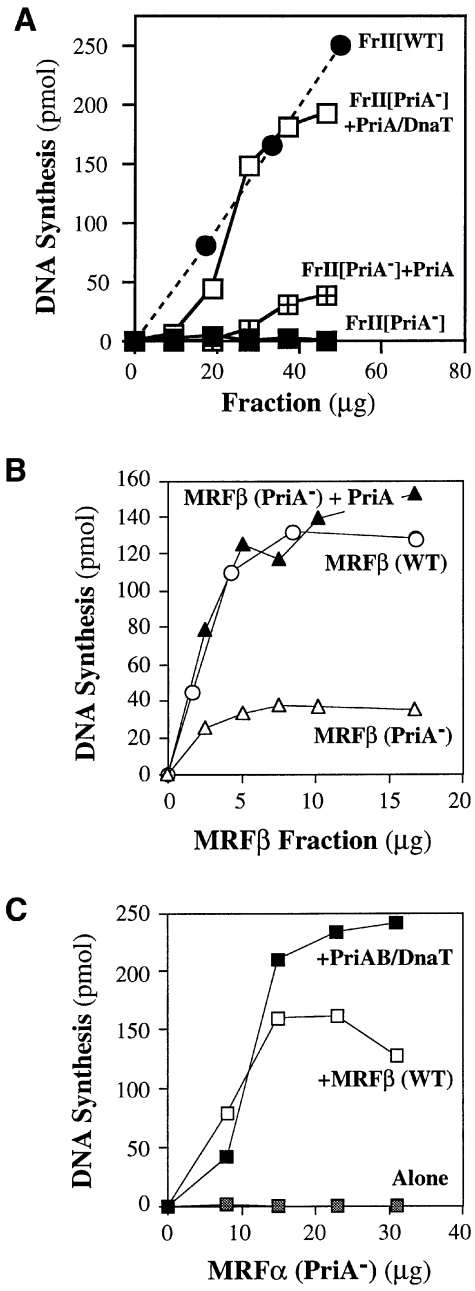

Fig. 2. Requirement for PriA and additional primosome proteins in the reconstituted Mu replication system. (**A**) Replication was catalyzed on STC1 (pXP10 target DNA) in the eight-protein system supplemented with the indicated proteins and with varying amounts of a crude enzyme fraction (fraction II) prepared from a PriA$^+$ (WT) or PriA$^-$ *E.coli* strain. (**B**) Replication was catalyzed on STC1 in the eight-protein system supplemented with MRFα, purified PriA, as indicated, and varying amounts of MRFβ prepared from PriA$^+$ (WT) or PriA$^-$ strains. (**C**) Replication was catalyzed on STC1 in the eight-protein system supplemented with MRFβ(WT) or purified PriA, PriB and DnaT, as indicated, and with varying amounts of MRFα(PriA$^-$).

The ϕX-type primosome supports initiation of semi-discontinuous DNA synthesis with initial preference for the Mu left end

Replication of full-length (37 kb) Mu DNA in induced lysogens proceeds semi-discontinuously (Higgins *et al.*, 1983), with DNA synthesis *in vivo* initiating 80–90% of the time at the left end of full-length Mu (Wijffelman and van de Putte, 1977; Goosen, 1978; Pato and Waggoner, 1987). However, initiation of mini-Mu replication *in vivo* takes place at the left end only ~50% of the time (Harshey *et al.*, 1982; Résibois *et al.*, 1982a,b, 1984). We examined these properties in the reconstituted Mu replication system. To distinguish between leading and lagging strand syn-

Table II. Requirement for ϕX-type primosome components and MRFα in cointegrate formation

Component omitted[a]	pmol[b]	Co (%)[c]
None	185	100
MRFα	0	<1
MRFα (ClpX and PriC added)	0	<1
PriA	0	<1
DnaBC	0	<1
PriB and DnaT	0	<1
PriB	37	20
DnaT	14	8

[a]The complete reaction mixture included STC1 (pXP10 target DNA), the eight-protein system, MRFα(PriA⁻), PriA, PriB and DnaT, with omissions as indicated. Where indicated, ClpX (7.6 μg/ml) and PriC (0.8 U/ml) were also included.
[b]Total deoxynucleotide incorporation (pmol) was determined by counting one-tenth of each reaction mixture.
[c]The remaining products were linearized with *Nde*I and resolved on a 0.6% alkaline agarose gel. The amount of cointegrates was quantitated by phosphorimagery. The level of cointegrates formed in the complete reaction (no components omitted), in which >95% of the strand transfer products were converted to cointegrates, was set arbitrarily at 100.

thesis and between initiation at the Mu left and right ends, STC1 was replicated in a six-protein system (the eight-protein system lacking DNA pol I and ligase) supplemented with MRFα, PriA, PriB, PriC and DnaT. Products were digested with a restriction enzyme that cleaves within the donor vector near the Mu left end (Figure 3A). Leading strands corresponding to initiation at the left or right ends as well as Okazaki fragments from lagging strand synthesis could be distinguished by size on a denaturing agarose gel. To ensure examination of leading and lagging strand synthesis associated with cointegrate formation, linearized cointegrate products were first purified from a native agarose gel prior to separation by denaturing gel electrophoresis.

We confirmed the presence of short products (1–3 kb) consistent with lagging strand synthesis in the isolated cointegrate products (Figure 3B), with leading and lagging strand synthesis accounting for roughly equal amounts of nucleotide incorporation. The addition of DNA pol I and ligase shifted all products to the unit length of the cointegrate (Figure 3C), supporting the conclusion that the short products were indeed Okazaki fragments. Quantitation of the products of leading strand initiation from the left and right ends in these isolated cointegrates revealed only a small bias for initiation from the left end.

The relative frequency of leading strand synthesis initiating at the left and right ends of mini-Mu was determined *in vitro* in this experiment from all replication products that had accumulated at the completion of the reaction (30 min) and *in vivo* in previous work (Résibois *et al.*, 1984) from all products that had accumulated late in development. To determine whether earlier replication products *in vitro* reflect the left end bias seen with full-length Mu *in vivo*, we examined the kinetics of initiation at the left and right ends. Reactions were allowed to proceed for 5–30 min, and products were digested with restriction enzymes that cleave in the donor vector either very near the Mu left (*Bam*HI) or right end (*Nde*I) to distinguish leading strands corresponding to initiation at the left or right ends on a denaturing agarose gel (see Figure 3A). Full-length products corresponding to leading strand synthesis across the entire mini-Mu element were first evident at 10 min. Quantitation of cointegrate products digested with *Bam*HI or *Nde*I (Figure 4A) revealed that 90–100% of cointegrates formed at 10 min corresponded to initiation at the left end of Mu (Figure 4B), indicating that the initial rounds of replication do reflect a left end bias. Products of right end initiation accumulated more slowly, so that by 30 min they accounted for 25–45% of the products (Figure 4B). Thus, some feature of STC3 or the DNA template may permit the replisome to be assembled more readily at the left end. All of these results indicate that Mu DNA replication reconstituted with the ϕX174-type primosome reflects characteristics of Mu DNA replication observed *in vivo*.

ϕX-type primosome constituents promote engagement of DNA pol III holoenzyme on the recombined substrate

Mu DNA synthesis can initiate without MRFα, MRFβ, DnaB, DnaC and DNA pol III holoenzyme on the deproteinized strand transfer product (Kruklitis and Nakai, 1994; Nakai and Kruklitis, 1995), especially when DNA pol I (or the Klenow fragment) is present at high levels (Figure 5B, lane 1). We determined whether DNA pol III holoenzyme (prepared from a UvrD⁻ strain so that it is not contaminated with helicase II) can catalyze Mu DNA synthesis on the deproteinized strand transfer product when PriA, PriB and DnaT are absent. The deproteinized template was incubated for 15–60 min in the six-protein system (in the absence of DNA pol I and ligase), and products were cleaved within the donor vector (Figure 3A) so that extension from the two ends could be distinguished. Even after 30 min, no DNA synthesis was catalyzed on the deproteinized template in the six-protein system alone (Figure 5A, lane 1). When the six-protein system was supplemented with high levels of the DNA pol I Klenow fragment, extension of the leading strand primers at both ends proceeded slowly, consistent with the low processivity and distributive action of pol I. These primers were extended only 0.2–0.4 kb by 15 min (Figure 5A, lane 2), gradually being extended 1 kb or more by 60 min (Figure 5A, lane 5). Few or no products corresponding to complete replication of the mini-Mu element were formed even after 60 min. Moreover, the same level of DNA synthesis was catalyzed if DnaB and pol III holoenzyme were not present together with pol I (Figure 5B, lane 1). These results indicate that DnaB and DNA pol III holoenzyme are not engaged on the deproteinized template under these conditions.

However, when PriA, PriB, PriC and DnaT were added to the reaction mixture that included DNA pol I, full-length cointegrates were formed in 30 min (Figure 5B, lane 2). DNA ligase was included in these reactions so that full-length cointegrates could be easily distinguishable from the shorter, 30 min extension products of DNA pol I (Figure 5B, cf. Co and Ex). Quantitation of cointegrate production revealed that under these conditions at least 90% of the cointegrate products were dependent on not only PriA and DnaT but also on the DnaBC complex and pol III holoenzyme (Figure 5C). In separate experiments, we determined that cointegrate production was dependent on both DnaB and DnaC when they were

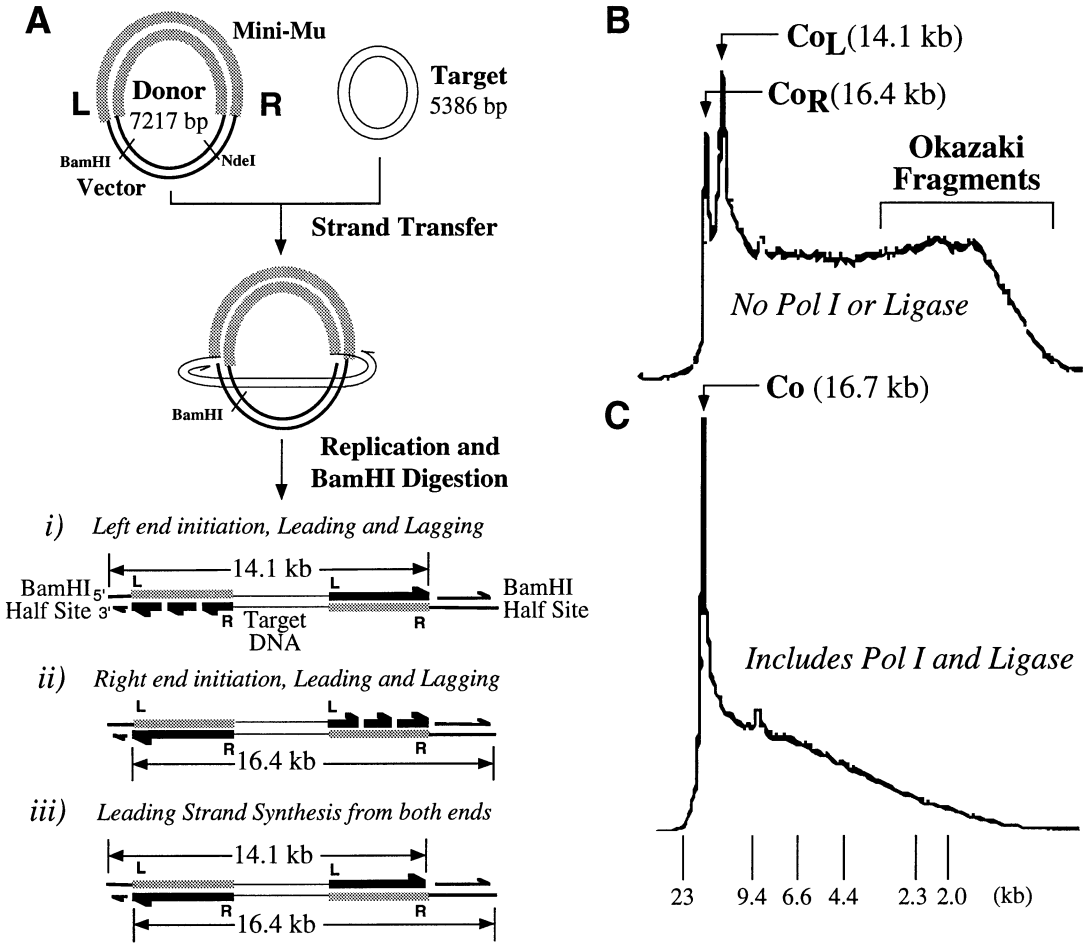

Fig. 3. Replication of STC proceeds by semi-discontinuous DNA synthesis. (**A**) *Bam*HI and *Nde*I cleave asymmetrically in the donor vector but not within the mini-Mu element or the transposition target. Cleavage of unligated replication products with one of these enzymes (e.g. *Bam*HI) results in a unique series of labeled DNA fragments whose lengths depend on the mode of replication: initiation of leading and lagging strand synthesis from the left (*i*) or right ends (*ii*) or initiation of leading strand synthesis from the primers at both ends (*iii*). (B) and (C) Replication on STC1 (ϕX174 RFI target DNA) was conducted in the six-protein system (lacking DNA pol I and ligase) (**B**) or the eight-protein system (**C**) supplemented with MRFα(PriA$^-$), PriA, PriB and DnaT. Full-length cointegrate products linearized with *Bam*HI were purified by native gel electrophoresis and then resolved on a 0.6% alkaline agarose gel, which was dried for phosphorimagery. Linear scans of the radiolabeled products in each lane are shown. Peaks corresponding to unit length cointegrate (Co), leading strand products resulting from initiation at the Mu right (Co_R) and left (Co_L) ends and products of lagging strands synthesis were identified based on their migration relative to molecular weight standards.

added individually (data not shown). Therefore, the PriA-dependent replication pathway engages DnaB helicase and pol III holoenzyme to replicate Mu DNA rapidly on the strand transfer product.

Extension of the leading strand primer by DNA pol I is not essential for PriA-dependent DNA synthesis on the Mu strand transfer intermediate

In pBR322 replication, an RNA polymerase transcript that primes DNA synthesis at the origin must be extended by DNA pol I to form a D-loop and expose a PAS on the displaced single strand to maximize PriA-promoted assembly of the pre-primosome (Minden and Marians, 1985). On the Mu strand transfer intermediate, there is no single-stranded region on the lagging strand side of each fork potentially to serve as a binding site for the pre-primosome (see Figure 7A). Although DNA pol I can extend the leading strand at each Mu end of the deproteinized template to expose single-stranded DNA, it was not essential for PriA-dependent cointegrate formation (Figure 5C). Its presence did increase the level of nucleotide incorporation and cointegrate formation by ~2-fold, suggesting the possibility that the efficiency of preprimosome assembly can be maximized by limited extension of the leading strand primers.

When DNA synthesis was catalyzed on STC1, the leading strand primers were not extended at all unless all required replication proteins including PriA, DnaT and MRFα were present (Figure 6, lane 1). When PriA or DnaBC was omitted, no cointegrates were formed, and the leading strand primers could not be extended by high levels of DNA pol I (Figure 6, lanes 2 and 3) as they were on the deproteinized template (lane 4). Whereas 400–500 nucleotides were incorporated per deproteinized template in 30 min, the amount of nucleotide incorporation during this time on the STC without PriA or DnaBC was below detectable levels, which correspond to <10 nucleotides being incorporated per template. This level of nucleotide incorporation by itself is unlikely to produce a duplex opening sufficient to promote primosome assembly. When the DNA duplex at a ColE1-type plasmid origin is opened by an R-loop, a single-stranded region with a

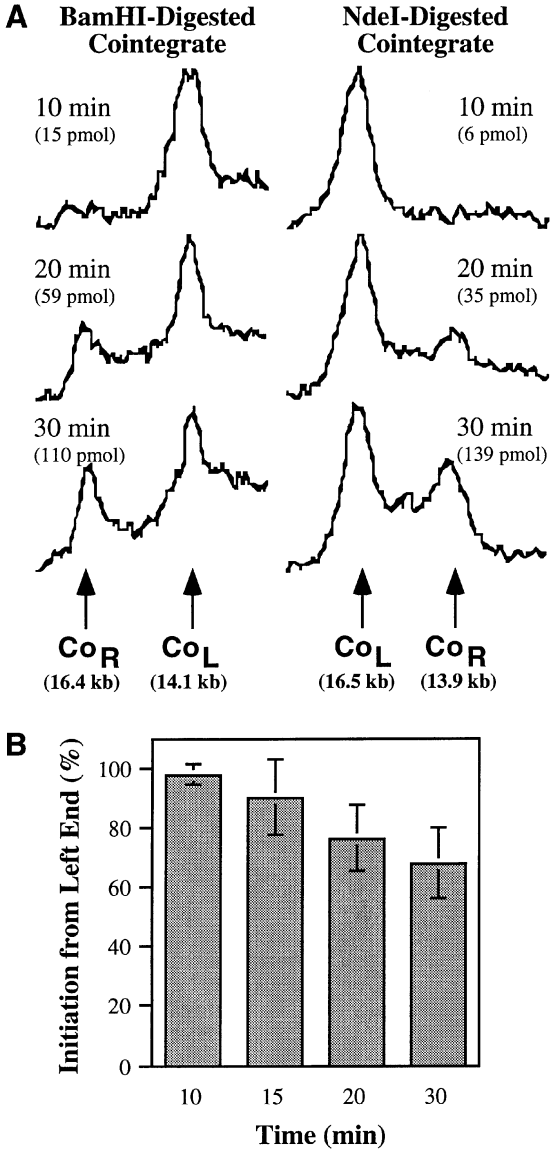

Fig. 4. Replication on STC initiates preferentially from the left end of Mu. (**A**) Replication on STC1 (ϕX174 RFI target DNA) was allowed to proceed for 5–30 min in the six-protein system (lacking DNA pol I and ligase) supplemented with MRFα(PriA$^-$), PriA, PriB and DnaT. Cointegrate products were linearized with *Bam*HI or *Nde*I and resolved on a 0.6% alkaline agarose gel, which was dried for phosphorimagery. Linear scans of the radiolabeled leading strand products from the 10, 20 and 30 min reactions are shown. Peaks corresponding to leading strand products resulting from initiation at the Mu right (Co_R) and left (Co_L) ends were identified based on their migration relative to molecular weight standards. Total deoxynucleotide incorporation (pmol) in each reaction is indicated; scans have been normalized for total cointegrate formation. (**B**) The percentage of total leading strand synthesis initiating at the Mu left end was quantitated by phosphorimagery. Results are the average of three independent trials, including one in which products were digested with *Nde*I and two in which products were digested with *Bam*HI; standard deviation of the mean is indicated by error bars.

minimum of 40 bases must be exposed to activate DNA synthesis in the absence of DNA pol I (Masukata *et al.*, 1987). Together with previous findings that the polymerase activity of DNA pol I is not required to initiate DNA synthesis on STC (Kruklitis and Nakai, 1994), our results indicate that the leading strand primer is not extended before assembly of the preprimosome on the STC and initiation of PriA-dependent Mu DNA synthesis.

Discussion

Mechanism for replisome assembly during Mu transposition

Bacteriophage Mu DNA synthesis by transposition requires a specific set of replication proteins (including DnaB helicase, DnaC protein, primase and DNA pol III holoenzyme) known to be required for initiation at *oriC* (Kaguni and Kornberg, 1984). Because initiation of Mu DNA synthesis does not require the DnaA protein (McBeth and Taylor, 1982; Kruklitis and Nakai, 1994), a major question has been how these proteins are assembled into a replisome once the recombination portion of the reaction has been carried out by the Mu transposition apparatus. The function of PriA, PriB and DnaT in Mu DNA synthesis characterized in this work and the previously characterized properties of the ϕX-type primosome indicate how these specific replication proteins are engaged for replicative transposition.

The transition from transpososome to replisome illustrates how the complex series of reactions needed for Mu replication are promoted sequentially through remodeling of nucleoprotein complexes at the Mu ends. STC1 is converted to STC2 by the action of the chaperone ClpX coupled to ATP hydrolysis (Kruklitis *et al.*, 1996), altering MuA quaternary structure (Levchenko *et al.*, 1995) and activating the transpososome's potential to promote transition to DNA replication. In a second ATP-dependent reaction, MRFα_2 displaces MuA in STC2 to form the prereplisome STC3, which only permits initiation of DNA synthesis by the specific group of replication proteins including MRFβ (Nakai and Kruklitis, 1995; Kruklitis *et al.*, 1996).

Our identification of MRFβ as PriA, PriB and DnaT makes evident the probable sequence of events that lead to replisome assembly for Mu DNA synthesis. In ϕX174 complementary strand synthesis, PriA binds to the PAS to begin the assembly process (Wickner and Hurwitz, 1975; Shlomai and Kornberg, 1980; Ng and Marians, 1996a). PriB and DnaT join the PriA–PAS complex, and then DnaB is delivered from the DnaB–DnaC complex to form the preprimosome (Ng and Marians, 1996a). Thus, PriA is the likely component that first assembles on STC3 or the deproteinized strand transfer intermediate, initiating the assembly sequence that leads to preprimosome assembly (Figure 7A–C). Our finding that PriA-dependent DNA synthesis on the deproteinized strand transfer intermediate could be catalyzed at lower levels without PriC or PriB was not surprising. PriC can be dispensable for primosome assembly and ϕX174 DNA synthesis (Ng and Marians, 1996a). Although PriB promotes interaction between PriA and DnaT, the PriA–DnaT complex on DNA can be formed at high DnaT concentrations in the absence of PriB (Liu *et al.*, 1996). DnaB in the preprimosome can recruit the two other specific enzymes needed to propagate the Mu replication fork. DnaB, through its specific interaction with the τ subunit of DNA pol III holoenzyme, can promote stable binding of this dimeric polymerase on the leading strand of the fork (Yuzhakov *et al.*, 1996), thus recruiting simultaneously

the polymerase for leading and lagging strand synthesis (Figure 7D). DnaB helicase can also attract primase (Tougo *et al.*, 1994) to initiate lagging strand synthesis (Figure 7E).

Our results indicate that PriA plays a crucial function in assembling a replisome on a recombination intermediate. A question raised by these studies is what constitutes a PAS on the Mu strand transfer intermediate. The pre-replisome STC3 allows only PriA-dependent Mu DNA synthesis to proceed, and the factors that play this gate-keeper role could stabilize a DNA structure that serves as a PAS. Even though these factors are not essential to engage PriA on this template, STC1 is replicated approximately twice as fast as the deproteinized template under identical reaction conditions (data not shown). Another important consideration is that the leading strand primers of STC3 cannot be extended to open the duplex prior to engagement of PriA. Thus, duplex opening at the Mu ends by DNA pol I cannot be the mechanism for creating a PriA-binding site. Instead, some feature of the DNA structure of a strand transfer intermediate may be important for initial PriA binding, which leads to duplex opening and primosome assembly. Recent evidence that PriA can bind to D-loops and DNA structures that resemble the branched structure of the strand transfer intermediate at each Mu end (McGlynn *et al.*, 1997) supports this hypothesis.

The left end bias observed in the initiation of Mu DNA replication *in vivo* and *in vitro* may reflect asymmetry of the STC in providing PriA-binding sites at the left and right ends. Such an asymmetry could be due to the presence of a strong PAS at or near the Mu left end. However, what would constitute a PAS on a branched recombination intermediate and how it may be structurally related to the PAS on the ϕX174 template are not yet clear.

Relevance to understanding the host system for coupling recombination with DNA replication

Kogoma (Asai and Kogoma, 1994; Kogoma, 1996) has hypothesized that DNA replication plays an important role in recombinant formation by homologous recombination and that the ϕX-type primosome plays a key role in assembling replisomes on recombination intermediates. Our results support this hypothesis and suggest that the Mu transposition apparatus ensures efficient replication of the Mu genome by specifically recruiting the host apparatus that links recombination with replication.

For replication linked to both Mu transposition and homologous recombination, replisome assembly would be coordinated with molecular events and signals different from those which control replisome assembly at *oriC*. While DnaA coordinates initiation with the cell cycle, our results indicate that PriA can respond to molecular signals on a recombination intermediate to initiate replisome assembly, a critical function in linking recombination with DNA synthesis.

In the Mu system, access of the potential replication forks to host proteins is carefully restricted. PriA can promote initiation only upon conversion of STC1 to STC3

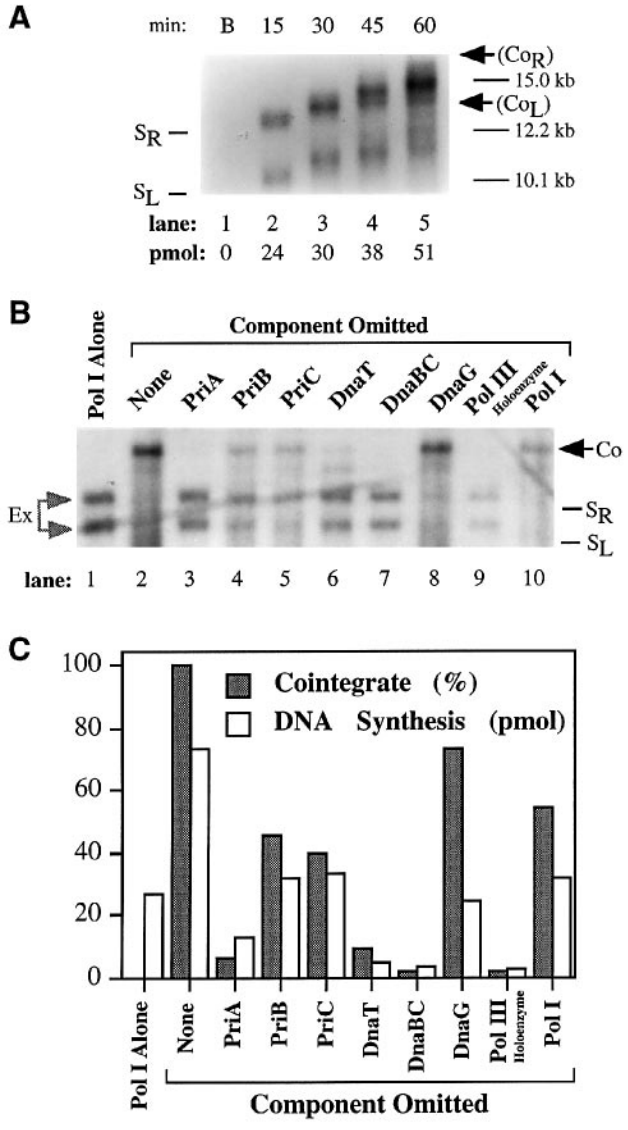

Fig. 5. ϕX-type primosome constituents promote engagement of DNA pol III holoenzyme on the deproteinized strand transfer product. (**A**) Replication was conducted on the deproteinized strand transfer product (ϕX174 RFI target DNA) in the six-protein system supplemented with the DNA pol I Klenow fragment (100 U/ml) for 15–60 min (lane 1: six-protein system alone, 30 min). Products were digested with *Bam*HI and resolved on a 0.6% alkaline agarose gel. The length of the replication products increases with time as the leading strand primers are slowly extended by Klenow. Total deoxynucleotide incorporation (pmol) in each reaction is indicated. For reference, the positions of unextended leading strand primers from the strand transfer intermediate (S) and of fully extended leading strands from the co-integrate (Co) resulting from initiation at the Mu left (Co_L, S_L) and right (Co_R, S_R) ends are indicated; replication products in this reaction did not reach full length. (**B**) Replication was conducted on the deproteinized strand transfer product (f1 RFI target DNA) in the eight-protein system supplemented with PriA, PriB, PriC, DnaT and additional DNA pol I (2 U/ml). Proteins were omitted as indicated (lane 1: replication by 2 U/ml DNA pol I in the absence of DnaBC, PriABC, DnaT and DNA pol III). Products were digested with *Eco*RI and resolved on a 0.6% alkaline agarose gel. Positions of the full-length cointegrate (Co), unreplicated strand transfer intermediates (S_L and S_R) and leading strand primers extended by DNA pol I (Ex) are shown. (**C**) Total deoxynucleotide incorporation (pmol) was determined by counting one-tenth of each reaction mixture (white bars). The remaining products were resolved on a 0.6% alkaline agarose gel. The amount of cointegrates was quantitated by phosphorimagery (shaded bars). The level of cointegrates formed in the complete reaction (no components omitted), in which ~60% of the strand transfer products were converted to cointegrates, was set arbitrarily at 100. Results are the average of two independent experiments.

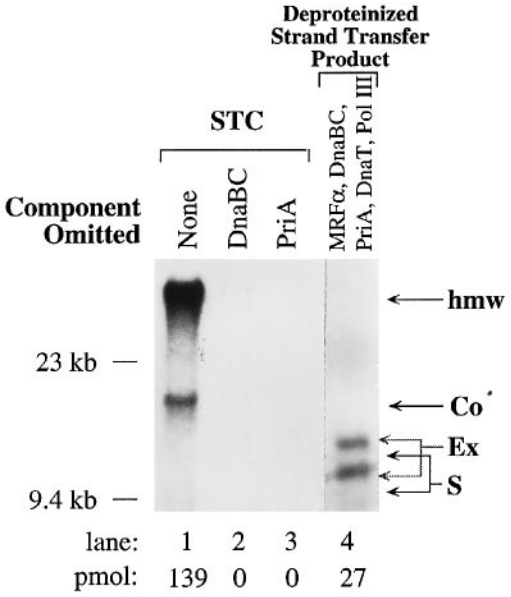

Fig. 6. Leading strand primers at the ends of Mu in STC are not extended in the absence of PriA. Replication was conducted on STC1 (f1 RFI target) in the eight-protein system supplemented with PriA, PriB, PriC, DnaT, MRFα(PriA⁻) and additional DNA pol I (2 U/ml). Proteins were omitted as indicated (lane 4: replication of deproteinized strand transfer product by 2 U/ml DNA pol I in the absence of MRFα, DnaBC, PriABC, DnaT and DNA pol III, 30 min). Products were digested with *Eco*RI and resolved on a 0.6% alkaline agarose gel. Positions of the full-length cointegrate (Co), unreplicated strand transfer intermediates (S) and leading strand primers extended by DNA pol I (Ex) are shown.

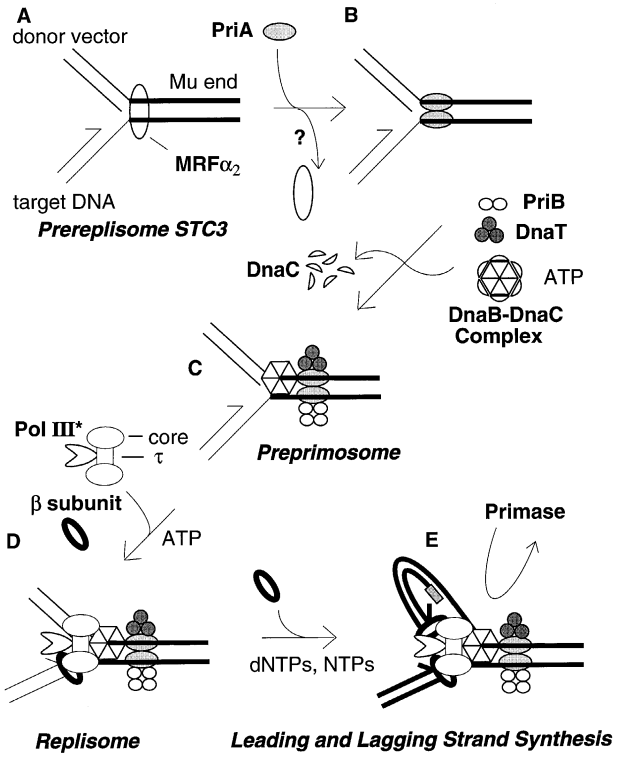

Fig. 7. Model for replisome assembly at the site of Mu strand exchange. Action of ClpX and MRFα$_2$ converts the transpososome STC1 to the prereplisome STC3 (**A**). In this complex, MuA has been removed from the Mu ends (one end is shown), forming a new nucleoprotein complex that does not permit the leading strand to be extended by DNA pol I (Kruklitis *et al.*, 1996). Even though there is no single-stranded segment on the lagging strand side of the fork, PriA binds to the pre-replisome (**B**), perhaps binding to a branched structure or a duplex opening stabilized by the prereplisome. Upon assembly of the preprimosome (**C**), DnaB promotes stable binding of DNA pol III* (holoenzyme minus the β subunit) to the leading strand primer (**D**) through interactions with the τ subunit (Yuzhakov *et al.*, 1996). The composition of the preprimosome is preserved (Ng and Marians, 1996b) as its helicase activity unwinds duplex DNA for leading strand synthesis. Its transient interaction with primase (Ng and Marians, 1996a) forms the primosome, catalyzing primer synthesis and initiating DNA synthesis by the lagging strand polymerase of dimeric pol III*.

by action of ClpX and MRFα$_2$ (Nakai and Kruklitis, 1995; Kruklitis *et al.*, 1996). This strategy may also be employed in homologous recombination. MRFα$_2$, which is involved in converting STC2 to STC3, may similarly be involved in controlling access of host proteins to D-loops, promoting PriA-dependent DNA replication. Not all homologous recombination requires PriA, suggesting that intermediates formed by strand exchange can be resolved with or without DNA replication (Kogoma *et al.*, 1996). Cellular factors may control the decision whether or not to assemble a replisome.

Thus, an intriguing question is how the engagement of PriA on a recombination intermediate would be regulated to control initiation. PAS sequences are underrepresented on the *E.coli* chromosome (Stuitje *et al.*, 1984), and at *oriM1*, the origin for iSDR in the *oriC* region, no PAS can be found by functional assays within the vicinity of ~2.5 kb (Stuitje *et al.*, 1984; Asai and Kogoma, 1994). It is therefore likely that signals other than the ϕX174-type PAS, DNA structures created during recombination and possibly stabilized by MRFα$_2$ or related cellular factors, play a key role in engagement of PriA. Through control of PriA action, the fate of a recombination intermediate can be determined, a process vital for the maintenance of the bacterial chromosome.

Materials and methods

Bacterial and bacteriophage strains and proteins

Escherichia coli strains EL500 (*priA1::kan, recD::mini-tet*), EL501 (pEL042 expressing wild-type *priA, recD::mini-tet*) and EL502 (pEL042, *priA1::kan, recD::mini-tet*) have been described (Lee and Kornberg, 1991). AT3327 *priA1::kan* and AT3978 *priA1::kan* were constructed by introducing *priA1::kan* into AT3327 (*mal*) and AT3978 (Hfr PK191 *his::*Mucts62pAp1), respectively, by P1 transduction. Mucts62pAp1, which carries a determinant for ampicillin resistance (Leach and Symonds, 1979), was grown by heat induction of AT3978.

DNA pol III* was purified from MGC1020 (W3110 *malE::Tn10, lexA3, uvrD::kan*) obtained from Dr Charles McHenry (University of Colorado Health Sciences Center) as previously described (Maki *et al.*, 1988). PriA, PriB, PriC and DnaT were purified from overproducing strains to >95% homogeneity as described (Marians, 1995). Purified preparations of these four proteins used for initial studies were kindly provided by Dr Arthur Kornberg (Stanford University School of Medicine). DNA pol I and the DNA pol I large (Klenow) fragment were purchased from New England BioLabs. All other proteins were purified as previously described (Kruklitis and Nakai, 1994; Nakai and Kruklitis, 1995; Kruklitis *et al.*, 1996).

Mu growth in vivo

To compare the plating efficiency of PriA$^+$ and PriA$^-$ bacterial strains, Mu *cts62*pAp1 was titered on various indicator strains which were seeded in soft agar on L broth plates. The number of p.f.u. per ml was determined after incubation of the plates overnight at 37°C. Relative plating efficiencies, with the titer on EL501 and AT3327 arbitrarily set to 1, were calculated from the averages of three independent trials; standard errors of the mean were <50%. To measure lysogenization frequency, indicator strains were infected with serial dilutions of Mu*cts62*-

pAp1, and cells were plated on L broth plates supplemented with ampicillin (50 μg/ml), incubated overnight at 30°C and scored for ampicillin-resistant colonies (Mu lysogens). Lysogenization frequency was calculated as the number of lysogens per p.f.u. Values shown are the average of three independent trials; the standard errors of the mean were <50%. Plating assays indicated that PriA⁻ strains had a 5- to 10-fold reduced viability relative to wild-type strains as observed elsewhere (Kogoma *et al.*, 1996); however, lysogenization frequencies were not corrected for this.

Mu DNA replication in vivo

To measure the level of Mu DNA replication by transposition *in vivo*, lysogens AT3978 (*his*::Mu *cts62*pAp1) and AT3978 *priA1::kan* were grown at 30°C to early log phase (OD_{600} = 0.4) and then incubated at 42°C until lysis occurred. Cultures were sampled at various times after the shift to 42°C. Cell growth in the samples was stopped by the addition of 10 mM sodium azide. RNase-treated genomic DNA from these samples (2.0 μg each) was digested to completion with *Eco*RI, separated on a 0.6% agarose gel (TAE electrophoresis buffer: 40 mM Tris, 20 mM acetic acid, 2 mM EDTA, pH 8.1), transferred to a nylon membrane (ICN Biotrans™) by alkaline capillary transfer (Selden, 1992) and probed with ^{32}P-labeled Mu DNA (500 000 c.p.m. per lane) from phage grown in *Proteus mirabilis*. The blot was stripped for 2 h at 75°C (1 mM Tris, 1 mM EDTA, 0.002% each of bovine serum albumin, polyvinylpyrrolidone and Ficoll 400, pH 8.0) and reprobed with ^{32}P-labeled pKA211 (from Dr Tsutomo Katayama, Georgetown University), which contains the *E.coli dnaA* gene located near *oriC* (Kornberg and Baker, 1992). Both probes were labeled to high specific activity (>2×10^8 c.p.m./μg) by nick translation (Sambrook *et al.*, 1989). The relative amplification of Mu over the *dnaA* gene was measured using the Molecular Dynamics Storm 840 phosphorimager system.

Mu DNA replication in vitro

Mu DNA synthesis was conducted on STC1 or the deproteinized strand transfer product (equivalent of 0.25 μg donor substrate), which was prepared as previously described (Nakai and Kruklitis, 1995) using pGG215 donor substrate (Surette *et al.*, 1987) and three different targets: pXP10 plasmid (Nakai and Kruklitis, 1995), ϕX174 RFI DNA and f1 RFI DNA (f1 contains no PAS; Zipursky and Marians, 1980). Where indicated, reaction mixtures (50 μl) contained crude *E.coli* enzyme fractions (fraction II) or fraction III of MRFα and MRFβ (240 U/ml of each unless otherwise indicated) prepared from *E.coli* strains WM433 (PriA⁺) or AT3327 *priA1::kan* (PriA⁻) as previously described (Nakai and Kruklitis, 1995). Purified proteins used in the reconstituted Mu replication system included PriA, PriB, PriC, DnaT (0.8 U/ml each; see Marians, 1995, for unit definition) and the eight-protein system composed of DNA gyrase (6.7 μg/ml), DnaB–DnaC complex (1.3 μg/ml), DnaG (0.84 μg/ml), DNA pol III holoenzyme (1.16 μg/ml), SSB protein (0.9 μg/ml), DNA pol I (0.2 U/ml) and DNA ligase (4 U/ml), or the six-protein system, which consisted of the same proteins except pol I and ligase. Reaction conditions and determination of total deoxynucleotide incorporation were as previously described (Nakai and Kruklitis, 1995). Reaction mixtures were incubated at 37°C for 30 min unless otherwise indicated. For quantitation of cointegrate production by phosphorimagery, reaction products were deproteinized and digested with *Bam*HI or *Nde*I (ϕX174 RFI target), *Nde*I (pXP10 target) or *Eco*RI (f1 RFI target). All of these enzymes cut once in the donor vector to linearize the cointegrate product. Products were then separated on a 0.6% alkaline agarose gel (Sambrook *et al.*, 1989).

For examination of leading and lagging strand synthesis and quantitation of initiation at the Mu left and right ends, the six-protein system was used and 1 mg/ml nicotinamide adenine mononucleotide (an *E.coli* ligase inhibitor) replaced nicotinamide adenine dinucleotide. This prevented the nick at the end of each leading strand from being sealed and prevented ligation of Okazaki fragments into a continuous strand. Products were deproteinized, digested with enzymes that cut once in the donor vector near either the left (*Bam*HI) or right (*Nde*I) Mu end and resolved on a 0.6% alkaline agarose gel. Where indicated, linearized cointegrates were first isolated on a native 0.6% agarose gel (TAE electrophoresis buffer) and purified using the GLASSMAX® DNA Isolation Matrix System (Gibco-BRL Life Technologies) before resolving on the alkaline gel.

Alkaline agarose gels were stained with SYBR® Green I nucleic acid stain for imaging and dried down for phosphorimagery on the Molecular Dynamics Storm 840 system. All quantitative data were analyzed using ImageQuant software. All images in the figures are from autoradiographs.

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