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Duplex Opening by Primosome Protein PriA for Replisome Assembly on a Recombination Intermediate

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PriA and other primosome assembly proteins of *Escherichia coli* recruit the major replicative helicase DnaB for replisome assembly during bacteriophage Mu transposition and replication. MuA transposase catalyzes the transfer of Mu ends to target DNA, forming a potential replication fork that provides the assembly site for the replisome. However, this fork lacks the single-stranded DNA needed to load DnaB. Although no pre-existing primosome assembly sites that bind PriA were found within the Mu end sequences, PriA was able to bind to the forked DNA structure created by MuA. The helicase activity of PriA could then open the duplex to create the DnaB binding site. In a tightly coupled reaction on synthetic forked substrates, PriA promoted both the unwinding of the lagging strand arm and preprimosome assembly to load DnaB onto the lagging strand template. PriA apparently translocated 3′ to 5′ along the lagging strand template until sufficient single-stranded DNA was exposed for binding of DnaB, which then translocated 5′ to 3′ in the opposite direction. Mutant PriA lacking helicase activity was unable to promote this process, and loss of PriA helicase impaired Mu DNA replication *in vivo* and *in vitro*. This suggests that the opening of the duplex by PriA helicase is a critical step in the initiation of Mu DNA replication. Concerted helicase and primosome assembly functions would allow PriA to act as initiator on recombination intermediates and stalled replication forks. As part of the replisome, PriA may act as a mobile initiator that minimizes interruptions in chromosomal replication.

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Introduction

Bacteriophage Mu DNA replication by transposition is a process intimately linked to non-homologous strand exchange catalyzed by MuA transposase. Monomeric MuA (Kuo *et al.*, 1991) binds to specific sites at the Mu ends (Craigie *et al.*, 1984), assembling into an active oligomeric transpososome bound to both Mu ends (Figure 1(a); Lavoie *et al.*, 1991; Mizuuchi *et al.*, 1992) aided by the host HU protein (Craigie *et al.*, 1985; Lavoie & Chaconas, 1993, 1994). Tetrameric MuA in the transpososome (Lavoie & Chaconas, 1990; Surette *et al.*, 1987) produces nicks at the Mu ends (Figure 1(b)) that are transferred to target DNA (Craigie & Mizuuchi, 1985, 1987) bound with a second transposition protein MuB (Adzuma & Mizuuchi, 1988). The resulting product, strand transfer complex 1 (STC1; Figure 1(c)), includes a potential replication fork (the Mu fork) that can act as the initiation site for Mu DNA replication at each Mu end, with the 3′-OH ends of target DNA providing the primers for leading strand synthesis. However, access to these forks is restricted by oligomeric MuA which remains tightly bound to the Mu ends (Kruklitis & Nakai, 1994).

In preparation for replisome assembly, MuA promotes the formation of a prereplisome at the Mu fork (Nakai & Kruklitis, 1995). First, the molecular chaperone ClpX alters quaternary interactions of oligomeric MuA (Levchenko *et al.*, 1995) in STC1, forming an altered transpososome STC2 (Kruklitis *et al.*, 1996). Additional host factors (Mu replication factor α_2 or MRFα_2) then displace MuA from STC2 to form a new nucleo-

Abbreviations used: STC, strand transfer complex; MRF, Mu replication factor; pol, polymerase; PAS, primosome assembly site; RF, replicative form; WT, wild-type.

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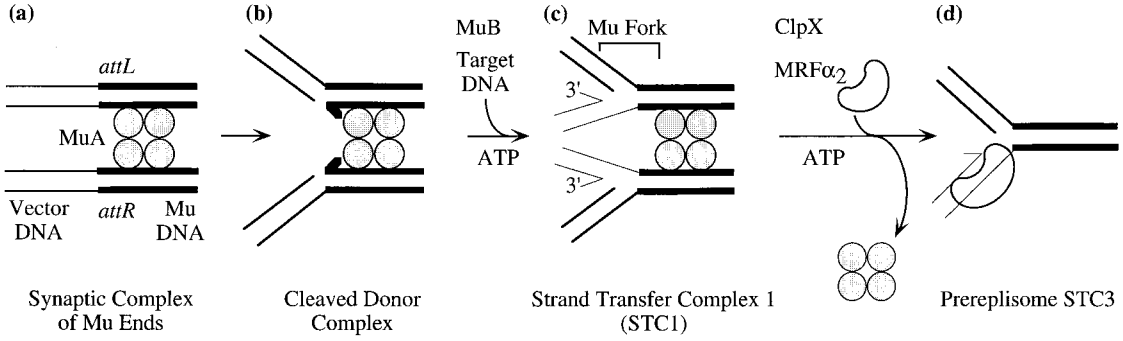


Figure 1. Formation of the transpososome and prereplisome during Mu transposition. (a) The transposase MuA binds to sites on the left (*attL*) and right (*attR*) ends of Mu (thick lines) forming a tetramer that brings the ends together in a synaptic complex. (b) The transpososome consisting of tetrameric MuA introduces a nick at each Mu end. (c) The resulting 3′-OH ends (half arrows) are transferred to target DNA (very thin lines) bound with MuB to form STC1, a complex which includes a potential replication fork (the Mu fork) at each Mu end. (d) Molecular chaperone ClpX changes the quaternary interactions within the MuA tetramer to form STC2 (not shown) and factors within the partially purified host fraction $MRF\alpha_2$ displace MuA from STC2, disrupting the synaptic complex of Mu ends and forming the prereplisome STC3 (only one Mu end is shown).

protein complex STC3 (Figure 1(d)), a prereplisome that permits Mu DNA synthesis only by specific primosome components and the DNA polymerase (pol) III holoenzyme (Jones & Nakai, 1997; Kruklitis *et al.*, 1996; Nakai & Kruklitis, 1995).

The disassembly of the transpososome leads to the formation of a replisome that catalyzes semi-discontinuous DNA synthesis from one Mu end to the other to form the cointegrate replication product. Central to this process is the assembly of a preprimosome at the Mu fork using the proteins PriA, PriB, PriC, DnaT, DnaB, and DnaC (Jones & Nakai, 1997). Together with primase these proteins comprise the ϕX174-type primosome, originally characterized as an apparatus that primes DNA synthesis on the single-stranded ϕX174 template (Wickner & Hurwitz, 1974). After the major replicative helicase DnaB is loaded from the DnaB-DnaC complex onto the lagging strand template, DnaB can serve as the organizing center of the replisome, stably binding the dimeric DNA pol III holoenzyme to the leading strand primer (Yuzhakov *et al.*, 1996). DnaB translocates 5′ to 3′ along the lagging strand template to unwind the helix (LeBowitz & McMacken, 1986) and attracts primase for lagging strand synthesis (Tougo *et al.*, 1994).

PriA, PriB, PriC, and DnaT's function in promoting the binding of DnaB at the fork distinguishes the process from DnaB assembly at the bacterial origin *oriC* where the initiator protein DnaA plays this role (Funnell *et al.*, 1987). PriA and the other primosome components have been found to play an important function in the initiation of DnaA-independent DNA synthesis such as replication of pBR322 (Minden & Marians, 1985). PriA-deficient strains assimilate genetic markers poorly by homologous recombination and are defective in double-strand break repair as well as inducible and constitutive stable DNA replication (Kogoma *et al.*, 1996; Masai *et al.*, 1994). Thus, PriA may couple homologous recombination to DNA replication by promoting replisome assembly at D-loop structures created by homologous strand exchange (Asai & Kogoma, 1994; Kogoma, 1996), a function that would also allow PriA to promote restart of DNA replication when replication forks stall.

PriA is the primosome component that initially binds to the DNA template. ϕX174 DNA includes a single primosome assembly site (PAS), a sequence to which PriA binds (Arai & Kornberg, 1981; Shlomai & Kornberg, 1980a), and there are two PASs near the origin of pBR322 (Zipursky & Marians, 1980). PriA can also bind to branched DNA structures that resemble D-loops (McGlynn *et al.*, 1997). We have previously demonstrated that PriA promotes primosome-dependent Mu DNA replication on both the prereplisome STC3 and the strand transfer product deproteinized by extraction with phenol (Jones & Nakai, 1997). However, the potential replication fork created by strand transfer does not include single-stranded DNA on the lagging strand arm. This poses a problem for the loading of DnaB, which occupies 20 nt of single-stranded DNA on the lagging strand template (Bujalowski & Jezewska, 1995). The 3′ to 5′ helicase activity of PriA (Lasken & Kornberg, 1988; Lee & Marians, 1987) could potentially create the necessary duplex opening, analogous to DnaA opening the duplex at *oriC* to create a binding site for DnaB (Bramhill & Kornberg, 1988).

Here, we investigate the function of PriA helicase in Mu DNA replication, establishing a new role for PriA's helicase activity in catalyzing a critical step in initiation. While the PriA helicase activity is not needed for primosome assembly on single-stranded templates (Zavitz & Marians, 1992), we demonstrate that it can open the duplex for entry of DnaB when sufficient single-stranded DNA is not available.

Results

PriA helicase can catalyze a critical step in the initiation of Mu DNA replication

PriA is essential *in vivo* for Mu DNA replication by transposition. No phage growth and no measurable amplification of Mu DNA can be detected in a *priA1::kan* host (Jones & Nakai, 1997). We determined that the mutant PriA K230R protein, which is defective in 3′ to 5′ helicase activity (Zavitz & Marians, 1992), was partially deficient in its ability to support Mu DNA replication. Mu plating efficiency was reduced by 50 % on a PriA K230R host (data not shown), and PriA K230R promoted relatively slow rates of Mu DNA replication *in vivo*. AT3853 *priA1::kan*, a thermoinducible Mucts62 lysogen, was transformed with a plasmid expressing either PriA (pEL042; Lee & Kornberg, 1991) or PriA K230R (pEL042 K230R), and the lysogens were induced at mid-exponential phase (1.5×10^8 cells/ml). Phage development was monitored by quantitating amplification of Mu DNA relative to a host marker by Southern blot analysis and by scoring phage production. In the PriA K230R strain, Mu DNA was replicated at a reduced rate and was amplified to less than 30 % the level attained in the PriA$^+$ strain (Figure 2(a)). Under these growth conditions, release of phage particles from the PriA K230R strain was delayed 20-30 minutes with a burst size approximately 50 % that of the PriA$^+$ lysogen (Figure 2(b)). When cultures were diluted 20-fold at the start of induction, the difference in phage yield between the PriA$^+$ and PriA K230R strains was decreased (Figure 2(c)). These results indicate that PriA helicase is required for optimal rates of Mu replication *in vivo*, especially for phage growth at higher cell densities.

The strand transfer complex STC1 can be converted to a cointegrate *in vitro* in a system containing the ϕX-type primosome components, DNA pol III holoenzyme, SSB, DNA gyrase, ClpX and the host fraction MRFα (Jones & Nakai, 1997). If PriA K230R replaced PriA in this system, cointegrate production would be reduced as much as 50-fold (Figure 3(a), cf. lanes 5 and 9). Other proteins present in a crude cell extract, however, could complement the helicase defect of PriA K230R. Both PriA and PriA K230R complemented an extract of AT3327 *priA1::kan* to promote high levels of cointegrate formation (Figure 3(b), lanes 3 and 4), consistent with our observation that phage growth can occur, albeit at a reduced rate, when PriA is defective for helicase activity. These results indicate that other host proteins can carry out the function performed by PriA helicase. How well they do so *in vivo* may be influenced by cell growth conditions such as cell density.

Both PriA and PriA K230R can bind to the forked DNA structure created by Mu strand transfer

Although PriA is needed to initiate Mu DNA replication, the type of PAS sequences that are on ϕX174 DNA and pBR322 could not be found at the Mu ends. We searched for ϕX-type PAS within the mini-Mu element of donor substrate pGG215 (Surette *et al.*, 1987; Figure 4(a)), which is readily converted to a cointegrate in the reconstituted Mu transposition and replication system. Denatured DNA fragments were assayed for their ability to

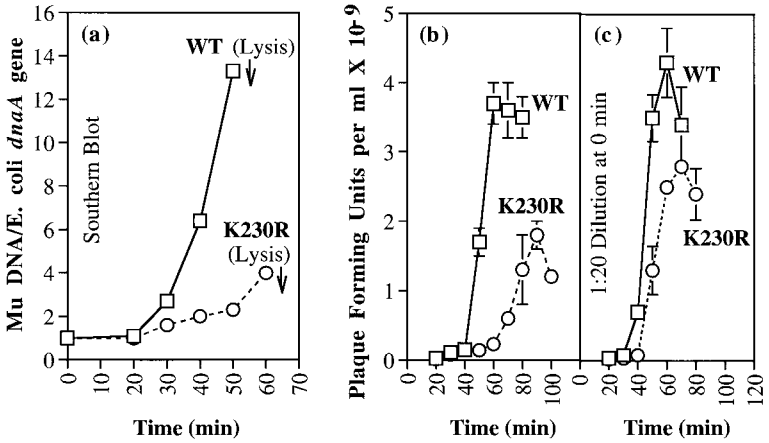


Figure 2. Deficiencies in Mu DNA replication supported by PriA K230R *in vivo*. (a) Mu DNA is amplified poorly during Mu lytic development in a PriA K230R strain (AT3853 *priA1::kan* pEL042 K230R) compared to a strain wild-type for PriA (AT3853 *priA1::kan* pEL042). A Southern blot of genomic DNA from samples collected at various points between induction (0 minute) and lysis was probed for Mu-specific and *E. coli*-specific (*dnaA* gene) sequences as described in Materials and Methods. The ratio of Mu signal/*E. coli dnaA* signal at 0 minute was set to 1. (b) and (c) Phage production is reduced in a PriA K230R strain but is improved by growth at lower cell density. Phage production was measured as described in Materials and Methods in cultures that were either left (b) undiluted or (c) diluted 20-fold at the point of induction (0 minute). Values are the average of three measurements with standard deviation of the mean shown by error bars.

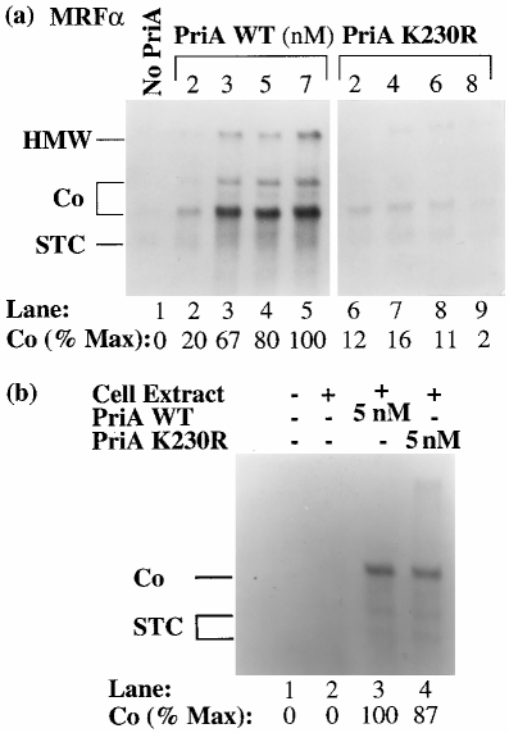


Figure 3. Differences between PriA and PriA K230R in their abilities to support Mu DNA replication *in vitro*. (a) PriA K230R lacks a function needed to initiate Mu DNA replication. Replication of STC1 in the reconstituted reaction system was conducted as described in Materials and Methods using PriA or PriA K230R as indicated. Autoradiographs of replication products are shown. In the most active reaction (lane 5) 90 % of STC1 was converted to cointegrate (Co); this level was set to 100 %. (b) The defective function of PriA K230R can be complemented by a crude cell extract (Fr II). Replication of STC1 supported by crude cell extract was conducted as described in Materials and Methods using PriA or PriA K230R as indicated. Autoradiographs of replication products linearized with *Eco*RI are shown. In the most active reaction (lane 3) >95 % STC1 was converted to cointegrate; this level was set to 100 %.

stimulate PriA's ATPase activity (Shlomai & Kornberg, 1980a,b; Zipursky & Marians, 1980), and the only PAS that were detected were the two present near the pBR322 origin (Figure 4(b), fragment D) located outside of the mini-Mu element. A donor substrate from which these two PAS were removed (pGG215ΔPAS) was active in Mu transposition and replication, using target DNA that also contains no PAS (data not shown). These results indicate that ϕX-type PAS are not required for Mu DNA replication.

PriA also binds to structures that resemble D-loops (McGlynn *et al.*, 1997), and this suggested that the PriA binding site may be created as the Mu ends are transferred to target DNA to form a branched DNA structure. In support of this hypothesis, band shift assays indicated that PriA binds to synthetic forked oligonucleotide substrates that mimic the DNA structure of the strand transfer product. A forked substrate containing the Mu right-end sequence was assembled from four oligonucleotides (Substrate A, Figure 5(a)). The duplex ahead of the fork consisted of 50 bp of Mu right end sequence with leading and lagging strand arms of 40 and 28 nt, respectively. As in the Mu strand transfer product, the fork is fully duplex except for a five-base gap between the leading strand primer and the fork. PriA was able to produce a discrete mobility shift with Substrate A (Figure 5(b), lanes 4-6), whereas it was unable to do so with the corresponding linear oligonucleotide that contains the Mu right-end (Substrate Z; Figure 5(b), lanes 1-3).

In addition, the deficiency of PriA K230R in promoting Mu DNA replication is not due to any defect in binding the Mu fork. PriA and PriA K230R bound to Substrate A (Figure 5(c)) with dissociation constants (K_D) of 21 and 19 nM, respectively, comparable to the K_D of 11 nM for binding PriA to the ϕX174 PAS (Ng & Marians, 1996a).

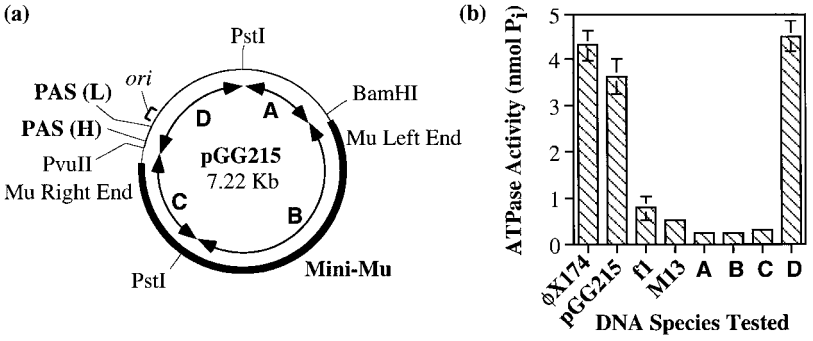


Figure 4. The Mu left and right ends do not contain PAS. (a) pGG215 donor substrate. The mini-Mu element is indicated in bold; the Mu left and right ends are on fragments B and C, respectively. The two pBR322 PAS (designated L and H; Zipursky & Marians, 1981) in the pGG215 vector are on fragment D. (b) Fragments that include regions of mini-Mu do not stimulate PriA's ATPase activity. Fragments A-D of pGG215 (subcloned into M13mp18) and full-length pGG215 were assayed for the ability to stimulate PriA's ATPase activity as described in Materials and Methods. Results are the average of three independent trials with standard deviation given by error bars.

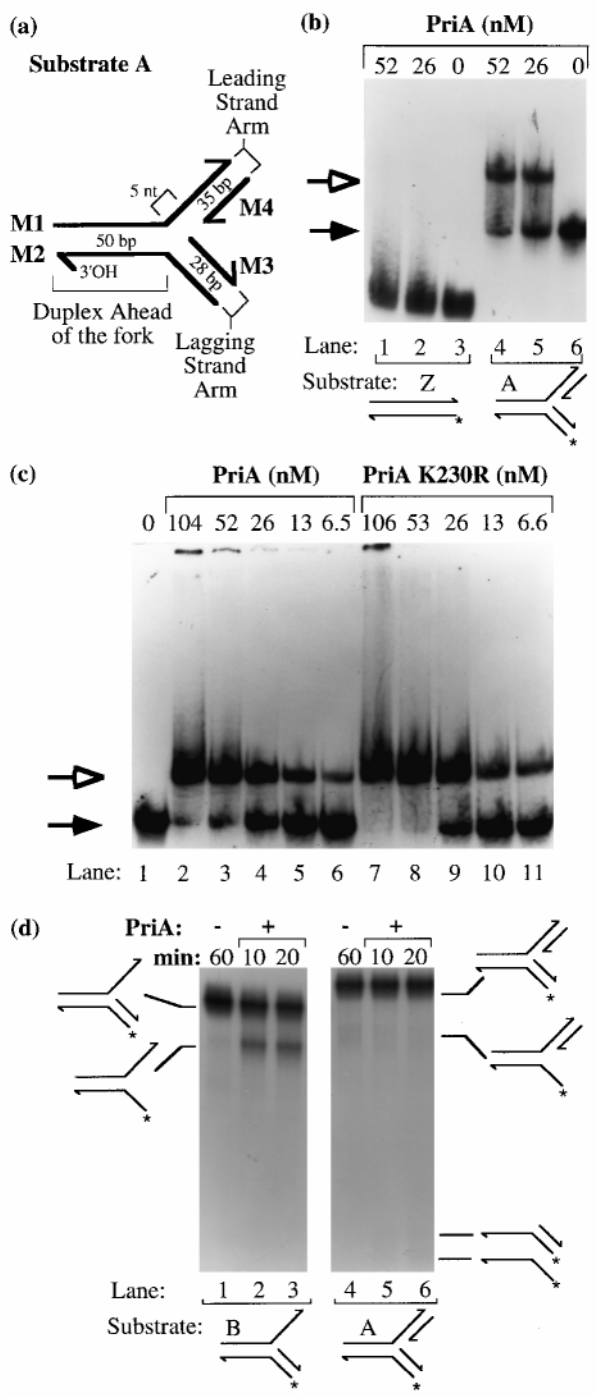


Figure 5. Ability of PriA to bind and unwind synthetic substrates resembling the Mu fork. (a) Substrate A (oligonucleotides M1, M2, M3, and M4). This substrate reflects the Mu fork DNA structure, with a five nucleotide (nt) opening present on the leading strand arm and a completely duplex lagging strand arm. (b) PriA binds to Substrate A but not to Substrate Z (oligonucleotides M2 and M5) in band shift assays. Band shifts were conducted as described in Materials and Methods. The filled arrow indicates the position of free Substrate A; the open arrow indicates the position of the shifted complex. (c) PriA and PriA K230R bind equally well to Substrate A in band shift assays. (d) PriA unwinds Substrate B (oligonucleotides M1, M2 and M3) more efficiently than Substrate A in helicase assays. Helicase assays that included PriA and SSB were conducted as described in Materials and Methods.

Unwinding of the lagging strand arm of a synthetic fork by PriA helicase

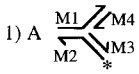
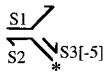
The role of PriA helicase in Mu DNA replication suggested that it may expose single-stranded DNA on the lagging strand side of a fork to load DnaB helicase. We investigated whether PriA could promote duplex opening at a fork as it promotes preprimosome assembly. Because PriA does not promote DNA synthesis as efficiently on the deproteinized strand transfer product as on the prereplisome STC3 (Jones & Nakai, 1997), naked DNA substrates with the exact structure of the Mu fork might not necessarily be the best substrates for PriA helicase. For this analysis we searched for suitable forked oligonucleotide structures that would serve as good substrates for PriA helicase but have insufficient single-stranded DNA to load DnaB onto the lagging strand template.

PriA helicase was more active on substrates with a single-stranded leading strand arm than on substrates with two duplex arms such as the Mu fork. Although PriA bound to both Substrate A (the Mu fork) and Substrate B (the analogous fork with single-stranded DNA on the leading strand arm) with nearly equal affinity (data not shown), its helicase was five times more active on Substrate B than on Substrate A (Figure 5(d), cf. lanes 1-3 with lanes 4-6; Table 1, lines 1 and 2). In the presence of SSB, the lagging strand arm (M2-M3 duplex) of Substrate B was unwound almost exclusively, most likely the result of PriA binding to the lagging strand template at the fork and translocating in a 3′ to 5′ direction.

A small gap may also facilitate access of PriA to the lagging strand template. PriA had little helicase activity on Substrate C (Table 1, line 3), a fork analogous to Substrate B but with a longer lagging strand arm and a different DNA sequence. Helicase action on Substrate C was comparable to that on Substrate B when a five-base gap was introduced on the lagging strand arm (Substrate C[− 5], Table 1, line 4). A gap of three nucleotides was sufficient to promote helicase action, whereas a gap of a single nucleotide was not (data not shown). PriA bound equally well to Substrates C and C[− 5] (data not shown), indicating that reduced helicase activity on Substrate C was not due to reduced binding affinity. Substrate C has a lagging strand arm (S2-S3 duplex) of 70 nt compared to 28 nt for Substrate B; in addition, it does not have Mu end sequences in the 30 bp duplex ahead of the fork (S1-S2 duplex). Although Substrate B has no single-stranded segment on the lagging strand template, some feature of Substrate B, such as the shorter length of the lagging strand arm or its DNA sequence, may permit exposure of single-stranded DNA on the lagging strand template, allowing PriA to initiate unwinding. The requirement of the gap in Substrate C for PriA helicase activity was not examined further at this time.

Our overall results indicated that Substrate C[− 5] was an ideal substrate for examining the

Table 1. PriA, DnaB, and preprimosome helicase activity on synthetic DNA fork substrates

Substrate	Proteins present	Total substrates consumed	Labeled products
1) A 	PriA, SSB	5%	3% 2%
2) B 	PriA, SSB	25%	**25%**
3) C 	PriA, SSB	3%	3%
4) C[-5] 	PriA, SSB	27%	**24%** 3%
5) C[-5]	PriA, PriB, PriC, DnaT, DnaBC, SSB	50%	**21%** 4% **25%**
6) C[-5]	PriA, PriB, PriC DnaT, SSB	43%	**39%** 1% 2%
7) C[-5]	PriB, PriC, DnaT, DnaBC, SSB	3%	2% 1%
8) C[-5]	PriA DnaBC, SSB	38%	**35%** 1% 2%
9) C[-5]	DnaBC, SSB	3%	3%
10) D 	DnaBC, SSB	31%	**31%**
11) D	PriA, PriB, PriC, DnaT, DnaBC, SSB	40%	**40%**

Helicase assays using the componenents indicated were performed as described in Materials and Methods. Oligonucleotide composition of each substrate is shown; the oligonucleotide designated with an asterisk is radiolabeled. Major products (>20 % of total substrate) are highlighted in bold; any potential products not listed represent <1 % total substrate. Less than 1 % of substrate was consumed in control reactions including only SSB.

role of PriA helicase during preprimosome assembly. The gap of five nucleotides and the single-stranded leading strand arm allowed preferential PriA helicase action on the lagging strand arm in the presence of SSB (Table 1, line 4), but the gap is too small to provide a binding site for DnaB (Bujalowski & Jezewska, 1995; Table 1, line 9). If DnaB is bound to the lagging strand arm of Substrate C[− 5], its single-stranded leading strand arm would also allow efficient unwinding of the duplex ahead of the fork by DnaB. When DnaB unwinds DNA in the absence of DNA pol III holoenzyme, a 3′ single-stranded tail is required on the DNA strand to be displaced (LeBowitz & McMacken, 1986) even when DnaB is acting as part of the preprimosome (Lee & Marians, 1989).

Duplex opening by PriA can promote loading of DnaB onto the fork during preprimosome assembly

The preprimosome (PriA, PriB, PriC, DnaT, and DnaBC) in the presence of SSB efficiently unwound the duplex ahead of the fork on Substrate C[− 5] (S1-S2 duplex; 29 % of the total substrate; Table 1, line 5). The major product of this process was single-stranded S2 (25 % of total substrate), a result of unwinding both the S1-S2 and S2-S3 duplexes. When DnaBC was omitted from the reaction mixture (Table 1, line 6), very little of the S1-S2 duplex was unwound, although the S2-S3 duplex was still unwound at high levels. Very little of the substrate was unwound at all if PriA was omitted from the reaction mixture (Table 1, line 7). These results are consistent with a mechanism in which PriA unwinds the lagging strand arm to promote unwinding of the duplex ahead of the fork by DnaB.

Preprimosome assembly was required for unwinding the S1-S2 duplex in this reaction. When PriB, PriC and DnaT were omitted, only the lagging strand arm could be unwound at high levels (Table 1, line 8). PriB, PriC, and DnaT bring PriA and DnaB together in a single complex (Liu *et al.*, 1996; Ng & Marians, 1996a,b). In contrast, PriABC and DnaT were not required for DnaB helicase action if the lagging strand arm of the fork was single-stranded. Substrate D, which has two single-

stranded arms, was unwound in the presence of DnaBC without the remaining preprimosome components (Table 1, cf. lines 10 and 11), provided that DnaB was allowed to bind to the substrate before the addition of SSB (data not shown).

We confirmed that PriA helicase activity was essential for unwinding of the S1-S2 duplex of Substrate C[− 5] by the preprimosome; little or no unwinding could be detected when PriA K230R replaced PriA (Figure 6(a)). In addition, we were able to distinguish participation of the two helicases by taking advantage of their different nucleotide requirements when ATP is not the major energy source (Lasken & Kornberg, 1988; LeBowitz & McMacken, 1986; Lee & Marians, 1987, 1989). Unwinding of the S1-S2 duplex of Substrate C[− 5] by the preprimosome components required both dATP and GTP to support the PriA and DnaB helicases, respectively, as well as low levels of ATP

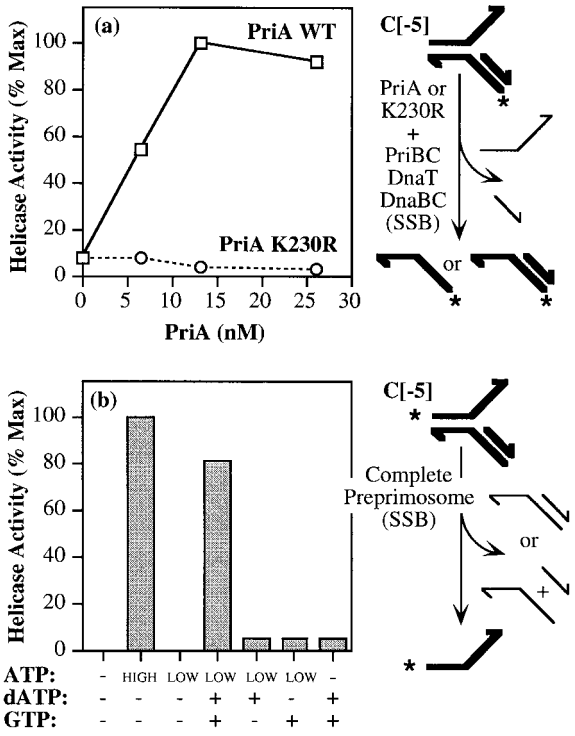


Figure 6. Contribution of the two preprimosomal helicases in unwinding forked substrates. (a) PriA K230R does not support unwinding of the S1-S2 duplex on Substrate C[− 5] by the preprimosome. Helicase assays that included PriA or PriA K230R as well as PriB, PriC, DnaT, DnaBC and SSB were performed as described in Materials and Methods. Total unwinding of the S1-S2 duplex was measured. The level in the most active reaction (13 nM PriA WT) was set to 100 %. (b) Nucleotide requirements for unwinding of the S1-S2 duplex in Substrate C[− 5] by the preprimosome. Helicase assays that included PriA, PriB, PriC, DnaT, DnaBC and SSB were supplemented with ATP (2 mM [HIGH] or 10 μM [LOW]), dATP (2 mM) and GTP (3.4 mM) as indicated. Production of S2 in the most active reaction (2 mM ATP) was set to 100 %. Results are the average of two independent trials; duplicate values varied by less than 5 %.

(⩽10 μM; Figure 6(b)). By itself this ATP concentration is insufficient to fuel S1-S2 duplex unwinding on Substrate C[− 5] (Figure 6(b)), but it probably plays a role in assembly or activation of the preprimosome to elicit its two helicase activities (Lee & Marians, 1989). In the absence of dATP needed to drive PriA helicase action, unwinding of the substrate was very low (Figure 6(b)), consistent with the inability of PriA K230R to promote unwinding of the S1-S2 duplex. In the absence of GTP, the S2-S3 duplex was unwound (data not shown), confirming that PriA could be driven by dATP, but only very low levels of S1-S2 duplex unwinding were observed (Figure 6(b)). In contrast, unwinding of Substrate D, which has two single-stranded arms, could be fueled by GTP alone (data not shown), consistent with the ability of DnaB to unwind this fork. These results demonstrate that both the 3′ to 5′ helicase of PriA and the 5′ to 3′ helicase of DnaB are needed to unwind the S1-S2 duplex on Substrate C[− 5] and that this process requires preprimosome assembly to promote the concerted action of the two helicases. This strongly suggests that unwinding of the S2-S3 duplex and the loading of DnaB onto the fork are coupled events.

Duplex opening by PriA is coupled to loading of DnaB during preprimosome assembly

We confirmed that unwinding of the S2-S3 and S1-S2 duplexes of Substrate C[− 5] by the preprimosome is tightly coupled using a competition experiment. The preprimosome components were first allowed to associate with this substrate at 0 °C, conditions that do not allow any helicase action (data not shown). Even after subsequent challenge with 100-fold excess competitor DNA (the single-stranded oligonucleotide M5), both duplexes were still unwound to form the single-stranded S2 product (Figure 7(a), open triangles). This level of competitor effectively inhibited action of PriA or the preprimosomal helicases if these proteins were not first allowed to associate with the substrate (Figure 7(a) and (b), filled symbols). Moreover, the DnaB helicase activity on Substrate D, which could be produced from Substrate C[− 5] by the action of PriA, was effectively inhibited by the competitor even when the substrate was first incubated together with DnaBC (Figure 7(c)). These results indicate that when the preprimosome components unwind the S2-S3 duplex of Substrate C[− 5], they promote binding of DnaB before running off the template, coupling the processes of duplex opening and preprimosome assembly.

Discussion

Role of PriA helicase in duplex opening and replisome assembly at the Mu fork

A universal step in the initiation of DNA replication is the opening of the DNA duplex to promote

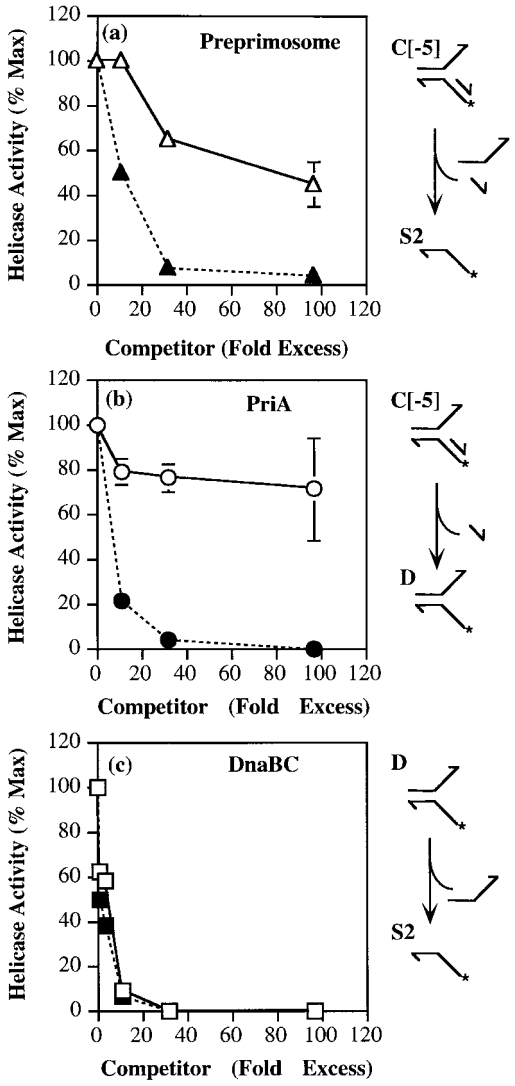


Figure 7. Duplex opening by PriA is tightly coupled to loading of DnaB onto forked substrates. Preprimosome components were initially incubated with substrate for ten minutes on ice before the addition of competitor DNA (the single-stranded oligonucleotide M5) and SSB followed by a 15 minute incubation at 30 °C (open symbols); alternatively, competitor DNA was present during the ten minute incubation on ice (filled symbols). Values are the average of multiple independent trials with standard deviation of the mean provided by error bars. (a) The preprimosome unwinds the two duplexes of Substrate C[− 5] in the presence of excess competitor. Helicase assays included preprimosomal components PriA, PriB, PriC, DnaT, and DnaBC. Production of S2 in the absence of competitor was set to 100 %. (b) PriA unwinds the S2-S3 duplex of Substrate C[− 5] in the presence of excess competitor. Helicase assays included the preprimosomal component PriA. The accumulation of Substrate D product was quantitated. Production of Substrate D in the absence of competitor was set to 100 %. (c) Helicase activity of DnaB on Substrate D is inhibited by challenge with competitor. Helicase assays included the preprimosomal component DnaBC complex. Production of S2 in the absence of competitor was set to 100 %.

binding of the major helicase DnaB, a process that ultimately leads to the assembly of the replisome. For bacterial chromosomal replication the DnaA protein serves the function of opening the duplex (Bramhill & Kornberg, 1988) and recruiting DnaB helicase to form the prepriming complex at *oriC* (Baker *et al.*, 1986; Funnell *et al.*, 1987). For initiation of Mu DNA replication by transposition, assembly proteins PriA, PriB, PriC, and DnaT of the ϕX-type primosome are involved in recruiting DnaB to the initiation site (Jones & Nakai, 1997). On synthetic DNA forks that have insufficient single-stranded DNA on the lagging strand arm to bind DnaB (as is the case with the Mu fork), the PriA helicase unwinds this duplex arm while promoting preprimosome assembly and binding of DnaB to DNA. Mu DNA replication *in vivo* proceeds at less than optimal rates when the PriA helicase is inactive, and PriA helicase is required for significant levels of Mu DNA replication *in vitro* in the reconstituted system, indicating that the PriA helicase can catalyze a critical step in initiation of Mu DNA replication. These results are consistent with a mechanism where PriA opens the duplex at the Mu fork to create a binding site for DnaB.

Our results indicate that during preprimosome assembly at a fork, DnaB binds to the same strand as PriA. The bidirectional helicase activity of the preprimosome was first demonstrated by assembling the complex on the phage ϕX174 PAS (Lee & Marians, 1989). However, the opposing helicase activities of PriA and DnaB raises the possibility that PriA at a replication fork might normally translocate 3′ to 5′ along the leading strand template, augmenting DnaB's progress on the lagging strand template. The role of PriA helicase at the Mu fork illustrates the utility of coupling two helicases moving in opposite directions on the same strand.

It is not yet clear how PriA would initiate helicase action on the Mu strand transfer product. Forked oligonucleotide substrates with the structure of the Mu fork are generally not good substrates for PriA helicase (e.g. Table 1, line 1). This is consistent with the observation that the preprimosome and DNA pol III holoenzyme initiate DNA replication less efficiently on the deproteinized strand transfer product than on the prereplisome STC3 (Jones & Nakai, 1997). There is also the problem of directing PriA to translocate along the lagging strand template rather than the leading strand template so that DnaB is loaded onto the correct strand. One possibility is that the prereplisome proteins ($MRF\alpha_2$) in STC3 promote PriA helicase action on the lagging strand arm of the fork. After completion of strand transfer, the transpososome is displaced by the prereplisome proteins with the aid of the molecular chaperone ClpX (Kruklitis & Nakai, 1994; Kruklitis *et al.*, 1996), and these proteins allow Mu DNA replication to proceed only by a PriA-dependent pathway.

In our current model for initiation at the Mu fork (Figure 8), PriA plays the function analogous

to that of DnaA at *oriC* by recognizing the initiation site created by strand transfer and opening the duplex for replisome assembly. We speculate that the prereplisome proteins present in STC3 (Figure 8(a)), while not required for PriA binding, may direct PriA to the lagging strand arm of the Mu fork. The binding of PriA to the Mu fork (Figure 8(b)) promotes recruitment of the other preprimosome proteins (Figure 8(c) and (d)). Initiation of PriA helicase action unwinds the lagging strand arm of the fork (Figure 8(d)), and once enough single-stranded DNA is exposed, DnaC dissociates from DnaB allowing DnaB to bind to the DNA (Funnell *et al.*, 1987; Learn *et al.*, 1997; Wahle *et al.*, 1989a,b). PriA and DnaB may then translocate in opposite directions on the lagging strand template (Figure 8(e)). However, the 3′ to 5′ helicase activity of the preprimosome requires significantly higher NTP concentrations than the 5′ to 3′ helicase activity (Lee & Marians, 1989), a property that may eventually cause PriA to disengage from the lagging strand template. Once DnaB is bound to the lagging strand template, DNA pol III holoenzyme can then assemble at the fork through its interaction with the primer-template and with DnaB (Yuzhakov *et al.*, 1996; Figure 8(f)), completing the assembly of the replisome. If PriA is defective in helicase activity, other helicases or a 5′ to 3′ nuclease could create a single-stranded segment on the lagging strand template for DnaB loading. But in such a mechanism, the process of duplex opening and DnaB loading would not be so tightly coupled, and the rate at which DNA replication is initiated may be relatively slow.

General function of the preprimosome and its two helicases in the replication of the host chromosome

A major question regarding the PriA helicase has been its function in cellular DNA replication and recombination and its relationship to PriA's role in primosome assembly. Knock-out mutations of the *priA* gene are not lethal but have serious consequences including slow growth, poor viability, sensitivity to DNA damaging agents, and characteristics of a constantly induced SOS response (Lee & Kornberg, 1991; Nurse *et al.*, 1991).

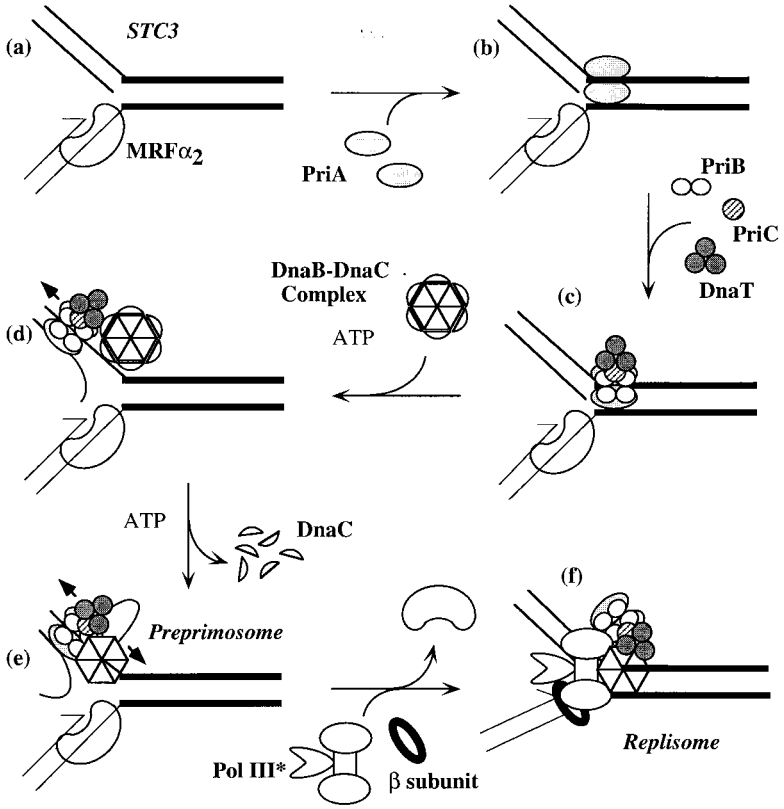


Figure 8. Model for PriA helicase-assisted assembly of the replisome during Mu transposition. (a) The prereplisome STC3 includes host protein components ($MRF\alpha_2$) which protect the leading strand primer. (b) PriA binds to the lagging strand template at the fork. (c) PriB, PriC and DnaT enter the PriA-DNA complex. (d) The DnaB-DnaC complex associates with the PriABC-DnaT complex, and the 3′ to 5′ helicase of PriA unwinds the lagging strand arm to create a binding site for DnaB. (e) With the exit of DnaC from the complex, DnaB is loaded onto the lagging strand completing assembly of the preprimosome. The opposing 3′ to 5′ and 5′ to 3′ helicase activities of the preprimosome could form a single-stranded loop on the template. (f) The association of DNA pol III holoenzyme with the leading strand primer-template and DnaB completes assembly of the replisome, with the hypothetical exit of $MRF\alpha_2$. PriA may eventually dissociate from the lagging strand template to terminate action of the 3′ to 5′ helicase.

Expression of PriA that is defective in helicase activity can restore the wild-type phenotype in essentially all respects (Zavitz & Marians, 1992), and helicase-deficient PriA proteins such as PriA K230R are fully active in promoting primosome assembly on the ϕX174 template (Zavitz & Marians, 1992). These data suggest that the primosome assembly function, but not the helicase function, plays a critical role in the replication and maintenance of the chromosome. Nevertheless, helicase mutants do not necessarily restore full transformation efficiency of pBR322-based plasmids (Zavitz & Marians, 1992) or full efficiency in inheritance of genetic markers by P1 transduction (Sandler *et al*., 1996). PriA helicase could accelerate the rate at which DnaB is loaded by expanding the duplex opening when insufficient single-stranded DNA is available.

It has been suggested that PriA together with proteins that promote homologous recombination may function in reassembly of the replisome when a replication fork stalls at a lesion or interruption in the template (Asai *et al*., 1994; Bierne & Michel, 1994; Courcelle *et al*., 1997; Kogoma, 1997; Kuzminov, 1995; Nurse *et al*., 1991; Rupp & Howard-Flanders, 1968; Zavitz & Marians, 1992). We have found that the preprimosome readily assembles on a forked substrate with a single-stranded leading strand arm, and such a substrate could result if DNA polymerase encounters a blockage on the leading strand template. If lagging strand synthesis continues uncoupled from leading strand synthesis, a single-stranded gap on the leading strand template would be created. Formation of such a product has been observed when DNA replication was reconstituted with eukaryotic cell extract on templates that have thymine dimers (Svoboda & Vos, 1995). Such single-stranded gaps created by DNA replication are thought to provide the SOS-inducing signal in *Escherichia coli* (Sassanfar & Roberts, 1990). On the resulting stalled fork, there may not be sufficient single-stranded DNA available on the lagging strand arm to allow restart of replication. While a nuclease or other helicase could potentially expose a region of single-stranded DNA, the most efficient method of creating the duplex opening is to couple PriA helicase action with preprimosome assembly.

The ability of PriA to bind to forked structures, open the duplex and promote primosome and replisome assembly is similar to the function carried out by the initiator DnaA at *oriC*. The major difference is that the signal to initiate replication for PriA is the DNA structure found at stalled forks and recombination intermediates. Once assembled, the protein composition of the preprimosome is conserved as the replisome translocates along the DNA template (Ng & Marians, 1996b). PriA in the replisome may facilitate restart of replication if the replisome encounters lesions or breaks in the DNA. PriA's ability to translocate in a direction opposite to DnaB may promote reopening of the duplex to reassemble the replisome as well as prevent disassembly of the preprimosome by allowing it to back off from the DNA lesion. While it is the task of DnaA to coordinate chromosomal replication with the cell cycle, the fully functional ϕX-type primosome would act as a mobile initiator that helps keep interruptions in the progression of the replication fork to a minimum.

Materials and Methods

Plasmids and bacterial strains

pND706-PriA was a gift from Nick Dixon (Australian National University); pND706-PriA K230R (described below) was used to overproduce PriA K230R. pEL042 (Lee *et al*., 1990) was a gift from Elliott Crooke (Georgetown University); this plasmid expresses PriA from its own promoter. To construct pND706-PriA K230R and pEL042 K230R, a single base substitution (A to G) at position 752 in the *priA* gene (Lee *et al*., 1990) was introduced into both plasmids using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene®) according to manufacturer's instructions. The mutagenized plasmids were sequenced by Veritas, Inc. (Rockville MD).

AT3327 and AT3327 *priA1::kan* have been described (Jones & Nakai, 1997). To construct AT3853 *priA1::kan*, the *priA1::kan* mutation (Lee & Kornberg, 1991) was introduced into AT3853 (Mucts62) by P1 transduction. Both *priA1::kan* strains were maintained on minimal media (Masai *et al*., 1994) supplemented with 0.2% (w/v) Casamino acids and 25 μg/ml kanamycin. Following $CaCl_2$ transformation (Sambrook *et al*., 1989) with pEL042 or pEL042 K230R, strains were routinely grown on LB (Sambrook *et al*., 1989) supplemented with 50 μg/ml ampicillin.

Mu growth *in vivo*

Plating efficiency on AT3327 *priA1::kan* transformed with either pEL042 or pEL042 K230R was determined as described (Jones & Nakai, 1997); results represent four independent trials. To examine the kinetics of phage production and Mu DNA amplification, 200 ml cultures of AT3853 *priA1::kan* transformed with either pEL042 or pEL042 K230R were grown to an A_{600} of 0.4 (1.5×10^8 cells/ml) at 30 °C, adjusted to a final concentration of 5 mM $MgSO_4$ and 0.2% (w/v) glucose, and then induced at 42 °C for 90 minutes. In some cases cultures were diluted 20-fold at the start of induction (0 minute). Phage production at various times postinduction was measured by plating dilutions of the cultures in duplicate with indicator bacteria (AT3327). Alternatively, genomic DNA from sampled cultures was subjected to Southern blot analysis, performed and quantitated as described (Jones & Nakai, 1997).

Proteins

All restriction enzymes, DNA pol I, *E. coli* DNA ligase, and T4 polynucleotide kinase were purchased from New England BioLabs. Purification of PriA K230R was essentially as described for PriA by Marians (1995) with the exception that a Sephacryl® S-200 HR HiPrep® 16/60 column (Amersham Pharmacia Biotech) was used for preparation of Fr IV. Concentrations of PriA and PriA K230R were determined by the method described by Pace *et al*. (1995). All other proteins, crude cell extract

(Fr II), and MRFα (Fr III) were prepared as described (Jones & Nakai, 1997).

Reconstituted Mu DNA replication assay

Replication of STC1 (50 fmol as complex) was carried out with 55 fmol DNA pol III*, 190 fmol (as monomer) DNA pol III β subunit, 1.2 pmol DnaG (as monomer), 130 fmol DnaBC complex ($DnaB_6$-6DnaC), 15 pmol SSB (as tetramer), 900 fmol gyrase (as gyrA-gyrB dimer), 8.4 pmol ClpX (as monomer), 900 fmol ClpP (as tetradecamer), 0.01 unit of DNA pol I, 1 unit *E. coli* DNA Ligase, 60 fmol PriB (as dimer), 130 fmol PriC (as monomer), 470 fmol DnaT (as trimer), PriA or PriA K230R as indicated, and crude cell extract (12 units) or MRFα (12 units) in a 50 μl reaction mixture as described (Jones & Nakai, 1997). Replication products were deproteinized and separated on 0.6% agarose gels in alkaline electrophoresis buffer (Sambrook *et al.*, 1989). Gels were neutralized and stained with 0.5 μg/ml ethidium bromide and then dried and subjected to phosphorimagery and autoradiography.

PriA ATPase assay

Fragments of pGG215 (Figure 4(a)) were subcloned into M13mp18 (Gibco BRL Life Technologies™), and replicative form (RF) DNA from these clones as well as ϕX174 RF, M13mp18 RF, f1 RF and pGG215 were used in PriA ATPase assays. Linear, double-stranded DNA was heated to 100 °C for ten minutes, then quickly cooled in an ice water-bath for five minutes prior to addition to the assay. The assay (15 μl total volume) was conducted in 50 mM Hepes-KOH (pH 8.0), 10 mM MgOAC, 1 mM DTT, 100 mM potassium glutamate, 0.1 mg/ml bovine serum albumin, 10 μg/ml rifampicin, 0.7 mM [γ-^{32}P]ATP (DuPont NEN®) plus 2 fmol (as duplex linear molecule) DNA, 170 fmol PriA (as monomer) and 15 pmol SSB (as tetramer). Reactions were incubated for 60 minutes at 37 °C, then stopped by the addition of 3 μl of 200 mM EDTA. A portion of each reaction (3 μl) was spotted on a PEI-cellulose thin layer chromatography plate (J.T. Baker) which was developed in 0.5 M LiCl, 4.6% (v/v) formic acid, dried and subjected to phosphorimagery. Generation of free ^{32}P phosphate was quantitated.

DNA substrates for band shift and helicase assays

Synthetic DNA substrates were constructed from the following oligonucleotides (Gibco BRL Life Technologies™): S1-CCATTAGCAAGGCCGGAAACGTCACC AATGCAACGATCAGCCAACTAAACTAGGACATCT-TTGCCCACCA; S2-CGCTACAGTCTGACGCTAA-AGGCAAACTTGATTCTGTCGCTACTGATTACGG TGCTGCTATCGATGGTTTCATTGGTGACGTTTC-CGGCCTTGCTAATGG; S3-AAACCATCGATAG-CAGCACCGTAATCAGTAGCGACAGAATCAAG-TTTGCCTTTAGCGTCAGACTGTAGCG; S3[-5]-ATCGATAGCAGCACCGTAATCAGTAGCGACA-GAATCAAGTTTGCCTTTAGCGTCAGACTGTAGCG; M1-GTTTTCGCATTTATCGTGAAACGCTTTCGCG-TTTTTCGTGCGCCGCTTCATGTACACCGTTCATCT-GTCCTCGTTCAAAGTTGGTCAGTT; M2-AAGCT-GTGGTGGTAACAAGTAGTGCCGGTGAAGCG GCGC-ACGAAAAACGCGAAAGCGTTTCACGATAAATGC-GAAAAC; M3-CCGGCACTACTTGTTACCACCA-CAGCTT; M4-AACTGACCAACTTTGAACGAGGAC-AGATGAACGGT; M5-GTTTTCGCATTTATCGT-GAAACGCTTTCGCGTTTTTCGTGCGCCGCTTCAC-CGGCACTACTTGTTACCACCACAGCTT. The oligonucleotide composition of each substrate is provided in appropriate Figure legends and Table 1. For each substrate, one oligonucleotide was radiolabeled with ^{32}P to a specific activity of 2×10^6 to 5×10^6 CPM/pmol using T4 polynucleotide kinase. This oligonucleotide (10 pmol) was combined with two- to fourfold excess of various unlabeled oligonucleotides in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 M NaCl, and the mixture was heated to 90 °C then slowly cooled to 40 °C. Annealed complexes were separated on 10% polyacrylamide gels (cross-linked at a ratio of 30:1) in TBE buffer (Sambrook *et al.*, 1989), and purified using the Elutrap® system (Schleicher & Schuell). The oligonucleotide composition of various substrates was confirmed by labeling all oligonucleotides in the purified substrate and separating them on a denaturing polyacrylamide gel.

Band shift assay

Band shifts were conducted essentially as described by McGlynn *et al.* (1997) using DNA substrates (16 fmol) and PriA or PriA K230R (0.13-1.1 pmol as monomer) in 20 μl reaction mixtures. Band shift gels were dried and subjected to phosphorimagery and autoradiography. The K_D value was determined as described by Ausubel *et al.* (1992) using the data shown in Figure 5(c).

Helicase assay

DNA substrates (16 fmol) were combined in 20 mM Tris-HCl (pH 7.5), 5.4 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml bovine serum albumin, and 2 mM ATP unless otherwise indicated (20 μl total volume) with the following proteins as indicated: 260 fmol PriA or PriA K230R (as monomer), 60 fmol PriB (as dimer), 2.2 pmol PriC (as monomer), 2.4 pmol DnaT (as trimer), 500 fmol DnaBC complex, and 240 fmol SSB (as tetramer). Reaction mixtures excluding SSB were incubated on ice for ten minutes. SSB was then added and reactions were incubated for 15 minutes (unless otherwise indicated) at 30 °C. Deproteinized products were separated on 10% polyacrylamide gels (cross-linked at a ratio of 30:1) in TBE buffer (Sambrook *et al.*, 1989) at 140 V for 2.5 hours. Gels were dried and subjected to phosphorimagery and autoradiography. All experiments included a negative control, a reaction mixture to which only SSB was added (e.g. Figure 5(d), lane 1), and markers representing potential helicase products. The percent of total substrate converted to each product was calculated.

Other

All quantitation was by phosphorimagery using the Molecular Dynamics Storm 840 system and ImageQuant™ 1.11 B15 software.

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