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Repair of transposable phage Mu DNA insertions begins only when the *E. coli* replisome collides with the transpososome

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Summary

We report a new cellular interaction between the infecting transposable phage Mu and the host *E. coli* replication machinery during repair of Mu insertions, which involves filling-in of short target gaps on either side of the insertion, concomitant with degradation of extraneous long flanking DNA (FD) linked to Mu. Using the FD as a marker to follow repair, we find that after transposition into the chromosome, the unrepaired Mu is indefinitely stable until the replication fork arrives at the insertion site, whereupon the FD is rapidly degraded. When the fork runs into a Mu target gap, a double strand end (DSE) will result; we demonstrate fork-dependent DSEs proximal to Mu. These findings suggest that Pol III stalled at the transpososome is exploited for coordinated repair of both target gaps flanking Mu without replicating the intervening 37 kb of Mu, disassembling the stable transpososome in the process. This work is relevant to all transposable elements, including retroviral elements like HIV-1, which share with Mu the common problem of repair of their flanking target gaps.

Introduction

Genomes of virtually all organisms harbor transposable elements (TEs) whose past as well as present activity continues to shape genome structure, function and evolution (Huang *et al.*, 2012). Active human TEs have been estimated to generate about one new insertion per 10–100 human births (Kazazian, 1999). In single individuals, a significant number of *de novo* insertions influence a range of phenotypes, both life enhancing (creating somatic heterogeneity in the brain; (Singer *et al.*, 2010, Perrat *et al.*, 2013)), and life threatening (primarily cancer-causing; (Kazazian, 2004, Mills *et al.*, 2007, Chenais, 2013)). Understanding the mechanism and regulation of these events is important for controlling their incidence.

The cutting and joining reactions of transposition that link the transposon to the target are well studied (Craig, 2002). The majority of DNA transposons, including retroviruses and retroviral-like transposons, transpose by a non-replicative mechanism i.e. without duplicating themselves in the process. These reactions leave short gaps in the target DNA on either side of the transposon. Transposition is not complete until these gaps are repaired. Yet this essential step is not as yet deciphered. Because a majority of these transposons are not duplicated, it is assumed that gap-filling polymerases fill the gaps, but conclusive evidence

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for a specific polymerase is lacking (Sasakawa *et al.*, 1981, Syvanen *et al.*, 1982, Yoder & Bushman, 2000). In this study we have used transposable phage Mu to investigate gap repair *in vivo*, because of unique features of Mu that make the analysis possible.

As a temperate transposable phage, Mu uses transposition to integrate into the *E. coli* host chromosome to generate a prophage during the lysogenic phase, and to amplify its genome over a hundred-fold during the lytic phase (Symonds *et al.*, 1987). During both phases, the chemical steps of transposition are the same: single-stranded DNA cleavages at Mu ends followed by strand transfer (ST) of the cleaved ends to phosphodiester bonds spaced 5 bp apart on the target DNA (Mizuuchi, 1992, Chaconas & Harshey, 2002). However, the structure of the Mu donor is different during the two phases, and has a bearing on how the ST intermediate is resolved (Fig. 1A and Fig. S1) (Harshey, 2015). During the lytic phase, Mu transposes from one site to another on the *E. coli* chromosome while remaining part of the covalently closed chromosome (Fig. 1B, 60 min). Here, the ST intermediate is resolved by target-primed replication by the Restart primosome, which fills the flanking target gaps while replicating across Mu (Fig. S1) (Nakai *et al.*, 2001). During the lysogenic phase, the infecting Mu genome is linear, is linked to several hundred base pairs of non-Mu flanking DNA (FD), and is non-covalently closed by the phage N protein (Fig. 1A) (Harshey & Bukhari, 1983, Puspurs *et al.*, 1983, Gloor & Chaconas, 1986). Here, the ST intermediate is resolved without replication (Akroyd & Symonds, 1983, Harshey, 1984, Liebart *et al.*, 1982, Chaconas *et al.*, 1983), during which the FD is degraded concomitant with gap repair (Au *et al.*, 2006). Using a convenient assay for monitoring FD degradation *in vivo*, we have learned that the first event in repair is removal of the FD by the RecBCD exonuclease (Choi *et al.*, 2014a), whose entry past the N-protein block is controlled by the transpososome and facilitated by ClpX (Choi & Harshey, 2010, Choi *et al.*, 2014b). *In vitro* experiments reveal that RecBCD action is required for stimulating endonucleolytic cleavage within the transpososome-protected DNA, leaving 4-nt flanks outside both Mu ends (Fig. 1A). This structure is likely the substrate for gap repair by host enzymes. The infection phase of non-replicative Mu transposition is an ideal system to investigate the gap repair process not only because of its high efficiency, where every infecting Mu genome integrates into the *E. coli* chromosome within 10 minutes, but also because we can track repair events using the FD degradation assay. We know that when integration of infecting Mu is blocked, the unintegrated N-linked Mu genome is indefinitely stable (Harshey & Bukhari, 1983, Puspurs *et al.*, 1983, Gloor & Chaconas, 1986). Thus, degradation of the FD is timed to coincide with some event that follows integration. We demonstrate in this study that this event is arrival of the *E. coli* replication fork.

Results

Repair of Mu insertions depends on the replication fork

The cutting and joining reactions of Mu transposition do not generate broken DNA ends (Mizuuchi, 1992), yet *E. coli* proteins that repair double strand breaks (DSBs) are required to recover Mu insertions after infection (Jang *et al.*, 2012). This suggests that one of the Mu target gaps might be converted to a break, perhaps by a replication fork. We will use the terminology DSE (double strand end) for the single DNA break created when a fork runs

over a gap, and DSB for DNA breaks generated spontaneously or introduced by restriction enzymes. We had envisioned two alternative scenarios for generating a DSE after Mu integration - either arrival of the native fork at the Mu insertion site, or start of replication on the FD-linked Mu insertion (Jang *et al.*, 2012). In *E. coli*, chromosome replication initiates at a unique origin *oriC* (Kornberg & Baker, 1992), where the initiator protein DnaA first recruits the DnaB helicase from the DnaB-DnaC complex, followed by loading of the Pol III holoenzyme (Fig. 1C, top). Bidirectional replication forks proceed from *oriC* to the terminus *ter*, but can collapse if they encounter nicks, gaps or other forms of DNA damage (Michel *et al.*, 2007). Collapsed forks are reassembled by several Restart pathways (Gabbai & Marians, 2010), the two main pathways requiring PriA and DnaT. The function of Restart proteins is similar to DnaA in recruiting DnaB for Pol III assembly (Fig. 1C, bottom). Mu uses the PriA Restart primosome to initiate replication specifically on Mu DNA during lytic growth (Fig. S1) (Nakai *et al.*, 2001).

To determine the role of the replication enzymes in the repair of Mu-linked FD, we selectively prevented replication forks initiating at *oriC* by using temperature sensitive (*ts*) mutants of DnaA and DnaC, or prevented Restart forks by using a PriA mutant, and monitored FD repair. At various times after Mu infection, *E. coli* genomic DNA (gDNA) was subjected to pulse-field agarose gel electrophoresis to separate integrated Mu from free Mu (Fig. 1D). The gDNA band was excised from the gel, and a PCR assay was used to detect Mu (primers 1, 2) and FD sequences (primers 3, 4) (Fig. 1E). In a wild type host infection, both Mu and FD are found integrated in the gDNA by 15 min, but the FD disappears soon after (Fig. 1E; WT). The FD is not degraded in a RecB mutant (Fig. 1E; RecB⁻), and degradation is delayed in a ClpX mutant, as reported earlier and (Fig. 1E; ClpX⁻) (Au *et al.*, 2006, Choi & Harshey, 2010, Choi *et al.*, 2014b). A different integration assay showed additionally that the FD was processed to a short length *in vivo* (Fig. S2), similar to that seen *in vitro* with RecBCD (Fig. 1A) (Choi *et al.*, 2014a).

When *oriC* replication is prevented in DnaA^{ts} and DnaC^{ts} mutants at 42°C, the *ts* mutants finish ongoing rounds of replication but do not re-initiate new ones (Wechsler & Gross, 1971), an observation we reconfirmed using FACS analysis for measuring chromosome ploidy, which shifts from two chromosome equivalents to one within ~ 60 min (Fig. 1F). Ninety minutes after replication inactivation at 42°C, the cells were infected with Mu. Both mutants supported Mu integration at 42°C (Fig. 1G; see Mu panels in the Dnats mutants), consistent with earlier data showing that Mu integration is independent of chromosome replication (Nakai & Taylor, 1985). However, in the absence of replication, the FD was not removed (Fig. 1G, compare FD panels at 30°C vs 42° in the *ts* mutants). The FD was degraded normally in the absence of PriA. These results were confirmed using an alternate assay, where the short product of FD degradation was not detected in non-replicating cells (Fig. S3). We conclude that chromosome replication is required for repair of Mu insertions, and that PriA-dependent replication initiating from Mu is not involved.

The entire Pol III replisome machinery is necessary for FD removal: gap-filling polymerases are not required

To determine if a specific component of the replisome is required for FD removal, *ts* mutants in all available components – helicase DnaB, β -clamp DnaN, β -clamp loader DnaX, and Pol III α subunit DnaE – were tested (Fig. 2A). Replication ceases immediately at 42°C in these replication elongation mutants (McMacken *et al.*, 1987). Mu integration was apparently normal when replication was arrested (Fig. 2B, 42°C and Fig. S4); however, FD degradation was blocked in all the strains only at 42°C.

E. coli has four other polymerases that assist in gap repair either during normal replication (Pol I), or during repair of DNA damage (Pol II, IV, V) (Fijalkowska *et al.*, 2012). Both Mu integration and FD degradation were similar to wild type in the single Pol I mutant as well as in the triple damage-inducible polymerase mutants (Fig. 2C). Thus, none of the gap-filling polymerases were required for Mu repair. These data do not address whether Mu integration might trigger an SOS response, the damage-inducible polymerases serving as a backup repair mechanism. To test this, we measured SOS induction by monitoring GFP fluorescence expressed from the promoter of the SOS-induced gene *sulA* with or without Mu infection in a wild type strain, or its *lexA3* and *lexA71* derivatives (Fig. 2D) (McCool *et al.*, 2004); *lexA3* is defective for SOS induction, and serves as negative control, while *lexA71* is constitutively induced. The data show that Mu infection does not induce an SOS response (Fig. 2D). We conclude that the Pol III holoenzyme, and not gap-filling polymerases, carries out Mu repair.

Permitting blocked forks to resume replication, resumes Mu repair

We interpret the above data to mean that interaction of the *E. coli* replication fork with the Mu transpososome is required to allow entry of RecBCD into linear FD ends that are normally protected by the phage N protein (Fig. 1A). To test this interpretation, Mu was first allowed to integrate in replication-blocked cells, followed by restart of replication either at the *oriC* site (DnaCts mutant) or at any elongation site (DnaEts mutant) on the chromosome, by a timed shift-down to 30°C. The DnaA_{ts} mutant was not used in these experiments because Mu can enter the lytic mode of growth in this mutant (Toussaint & Faelen, 1974, McBeth & Taylor, 1982), during which the Restart fork would replicate Mu, bypassing FD repair, as also seen in RecBCD and other mutants which permit Mu lytic growth without FD repair ((Choi & Harshey, 2010, Choi *et al.*, 2014a); see also Fig. S3).

In the absence of chromosome replication, the FD remained stable at 42°C when monitored across a 2 hr window in both DnaC and DnaE mutants (Fig. 3A, top panel; 120 min time-point not shown). When replication-blocked cells harboring integrated but unrepaired Mu in their genomes were shifted down to 30°C after 15, 30 and 60 min at the non-permissive temperature, the FD was degraded in both mutants within 15–30 min after the shift-down (Fig. 3A, bottom 3 panels).

For *E. coli* growing in a rich medium, it takes ~40 min for the bi-directional fork to travel from *oriC* to *ter* (Fig. 3B) (Cooper & Helmstetter, 1968). Since Mu integration events occur all over the *E. coli* chromosome (Manna *et al.*, 2004, Ge *et al.*, 2011), on average, the fork

would encounter a Mu insertion in approximately half that time, consistent with the average observed time for FD degradation. We conclude that repair of Mu insertions does not begin until the replication fork arrives at the Mu site.

FD is degraded by replication forks initiating at *oriC*-independent sites

Does a fork have to initiate at *oriC* to promote events that result in FD repair? To address this question we used an RNase HI mutant (*mhA*), where *oriC*-independent initiation can occur from RNA-DNA hybrids (R-loops) at multiple ectopic *oriK* sites using the PriA pathway (the R-loops are normally removed by RNase HI; (Kogoma, 1997)). The mutant strain grows poorly because of fork collisions associated with unregulated replication, and because of an SOS-constitutive phenotype brought on by DNA breaks (Fig. 4A, B) (Maduiké *et al.*, 2014). The *DnaA_{ts}* mutation was moved into this strain to additionally prevent *oriC* replication; the double mutant grows even more slowly (Fig. 4A). Nonetheless, both mutants still supported Mu integration at 30°C as well as at 42°C, and although slightly delayed, the FD was clearly degraded at both temperatures (Fig. 4C). We conclude that the FD degradation takes place in the presence of replication forks even when replication is not initiated at *oriC*.

Release of the replication block generates Mu-proximal DSBs

When the replication fork runs into the Mu transpososome, it is expected to stall because the Mu transpososome is extremely stable (Surette *et al.*, 1987, Choi *et al.*, 2014a). If the fork traverses the Mu gap on one strand, a DSE would be generated on the other strand. To test for the presence of the expected DSE at the junction of Mu and the replication fork, we marked both Mu and the potential DSE with fluorescent markers. Mu was fluorescently labeled by first incorporating a TetO array into the prophage genome (Fig. 5A), such that phage viability was not affected; similar phage titers were obtained upon induction of both wild type and TetO-engineered prophages (Fig. 5B, C). When the Mu::TetO phage were used to infect cells expressing TetR-mCherry at an MOI of 1, > 80% of the cells had single mCherry foci (Fig. 5D, E).

DSEs were fluorescently labeled using two different proteins, Mu Gam and RecA. Mu Gam binds directly to a DSE (d'Adda di Fagagna *et al.*, 2003), while RecA binds to the single-strand region of a processed DSE (Cox, 2001, Dillingham & Kowalczykowski, 2008). Fluorescent fusions to these proteins bind to DSEs *in vivo* (Shee *et al.*, 2013, Renzette *et al.*, 2005). Since overexpression of Gam interferes with repair of DSEs and cell viability (see Fig. S5), its expression was controlled by the tightly regulated rhamnose-inducible promoter in pGam-GFP, repressing it with 0.2% glucose until needed; rhamnose was added only 30 min before Mu infection. RecA-GFP is expressed constitutively from its normal chromosomal location.

When cells expressing TetR-mCherry and Gam-GFP were infected with Mu::TetO at an MOI of 1 where the majority of cells had an mCherry focus (Fig. 5), < 0.1% of the cells had visible GFP foci before infection; however, their numbers increased at least 30-fold after Mu infection (Fig. 6A). The green foci were binned into different categories with respect to their proximity to the red foci. Of the foci that appeared after Mu infection, ~ 40% either co-

localized with or were proximal to Mu (Fig. 6A, right). The overall low numbers and low intensity of Gam-GFP foci may be due to limiting its expression to only 30 min prior to Mu infection, which was necessitated because prolonged expression had a negative impact on cell viability (Fig. S5).

When RecA-GFP was used to monitor DSEs, the GFP foci were bright and 24% of uninfected cells had a single focus, consistent with the frequency of RecA foci detected in cells growing in rich media (Fig. 6B) (Renzette *et al.*, 2005). Infection with Mu increased their numbers by 13%. Here, >50% of the total number of the foci seen after infection were associated with Mu, suggesting that the majority of the Mu-induced RecA foci are near Mu. That only a fraction of the cells with Mu foci had Gam or RecA foci, could be due to fast repair of the DSE. This inference is supported by the observation that Mu infection does not induce the SOS response (Fig. 2D); induction of an SOS response requires that the DSE persist (Kuzminov, 1999, Lusetti & Cox, 2002). GFP foci not associated with Mu might be accounted for recession of the DSE by RecBCD to large distances from the site of the break (Shee *et al.*, 2013).

To test if appearance of the GFP foci was dependent on replication, replication-arrested cells (*DnaEts*) were infected with Mu in cells expressing Gam-GFP. New GFP foci appeared only when the replication block was removed (Fig. 6C and S6); 50% of these foci were associated with Mu. (This experiment could not be conducted using RecA-GFP because of a high background of GFP foci in the *DnaEts* strain at 42°C (Massoni & Sandler, 2013, Renzette *et al.*, 2005)). The independent probability of localization of the GFP foci next to Mu-mCherry foci was estimated to be less than 0.6%, a number much lower than the observed 50% co-localization (see Fig. 6 legend).

We conclude that Mu insertions generate DSEs in their vicinity that are dependent on chromosomal replication.

Discussion

This work opens a new window into the repair of transposon insertions, the majority of which share with Mu the problem of repair of flanking target gaps. Progress in this area of transposon research has languished likely because of the difficulty of studying the repair of such short gaps. Mu has provided the first insight into this problem primarily because of the unusual feature of long FD sequences linked to the infecting Mu genome, which are repaired concomitant with gap repair (Fig. 1A). The development of a convenient *in vivo* assay for monitoring FD repair (Au *et al.*, 2006), has allowed us to make large strides into understanding the repair process, both *in vivo* and *in vitro*. *In vivo*, the RecBCD exonuclease was found to be responsible for removal of the FD only after Mu integration. Thus, some post-integration event(s) must allow RecBCD access by releasing the N protein protecting the FD (Choi *et al.*, 2014a). *In vitro*, a strand transfer substrate with FD lengths similar to the *in vivo* substrate but lacking N, showed that RecBCD degrades the FD until it encounters the transpososome, leaving 19 bp of FD, which is shorted to 4 nt with added cell extracts (Choi *et al.*, 2014a). The present study confirms the generation of the short FD product *in vivo* (Figs. S2 and S3). The major finding of this study is that the event that triggers post-

integration FD removal is arrival of the *oriC* replication fork at the Mu insertion site (Figs. 1–4). Thus, proximity of the replisome to the transpososome is required to initiate N removal. A requirement for the native fork explains why the DSB repair machinery is required to recover Mu insertions (Jang *et al.*, 2012). We show that fork-dependent DSEs appear proximal to Mu (Fig. 6), and establish that gap-filling polymerases are not required for Mu repair (Fig. 2).

Model for Pol III-mediated coordinated repair of flanking target gaps without replicating the intervening Mu DNA

Why does Mu depend on the Pol III replisome and not gap-filling polymerases, and why does it employ a repair scheme that generates additional DNA damage for repair? We suggest that the Mu transpososome partners with the replisome for multiple tasks. First, the transpososome uses the incoming Pol III to signal just-in-time FD degradation, protecting the flanking gaps until the polymerase is on site to fill these gaps. Next, Pol III stalled at the transpososome is exploited for coordinated repair of both target gaps flanking Mu without replicating the intervening 37 kb of Mu. Finally, the replisome is used for transpososome disassembly concomitant with gap repair, as discussed below.

According to the data in Figs. 2–6, replisome interaction with the transpososome is somehow sensed at the distant N protein, destabilizing/removing N to allow RecBCD entry (Fig. 7A). At the same time, Pol III is in position for its leading strand subunit to fill the proximal Mu gap. However, the fork stalls because the transpososome is blocking its path. The Pol III subunit on the lagging strand gets dislodged while remaining attached to the leading strand subunit via the clamp loader (Fig. 7B; the DSE may not be released as yet, but is shown as such to facilitate demonstration of the next step). The dislodged subunit is now available to reload on the target DNA flanking the distal Mu gap, which although 37 kb downstream, is actually proximal to Pol III because of a hairpin bend (140°) in the target DNA held within the transpososome (Montano *et al.*, 2012). As the polymerase moves forward to fill both gaps, the transpososome must be removed because the gaps are protected within it (Lavoie *et al.*, 1991, Mizuuchi *et al.*, 1991). Such a replisome-transpososome partnership ensures that the gaps are not exposed prior to transpososome disassembly, that gap repair is synchronized at both ends, and that gaps are filled without having to replicate the entire length of the intervening Mu DNA (non-replicative transposition). The remaining 4 nt of FD must be trimmed by a nuclease prior to sealing the DNA with ligase. The lagging strand DSE is subsequently repaired by homologous recombination, and the stalled replication fork reinstated by the Restart machinery (Fig. 7B). DSE repair is obligatory for cell viability, since absence of the recombination-restart proteins was seen to lower recovery of Mu insertions by two orders of magnitude (Jang *et al.*, 2012).

The co-ordinate gap repair model we propose, in which Mu is not replicated, is consistent with the observation that Mu is not replicated immediately after integration (Harshey, 1984, Liebart *et al.*, 1982). We note that when the DSE generated at the Mu gap is repaired, and the stalled fork eventually resumes its travel, Mu will be replicated as a normal segment of the chromosome and inherited by both daughter cells. An alternative model where the gaps are not co-ordinately repaired will have to invoke two DSEs at each end: repair-restart at the

first DSE, replicate through Mu, repair-restart at the second DSE. The model we propose is parsimonious, reasonable and testable.

Why has Mu evolved an elaborate mechanism for removal and repair of the FD given that it can bypass FD repair to enter lytic growth in DnaA, RecBCD and some MuA mutants (this work and (Choi & Harshey, 2010, Choi *et al.*, 2014a)). We believe that a mechanism limiting Mu replication to short gaps at the Mu ends gives the phage a chance to enter the prophage state.

Repair of other transposon insertions

The short gaps generated in the target are a universal feature of transposition. The 4 nt FD overhangs in Mu are similar to 3 nt or 2 nt overhangs on the 5' flanking DNA of transposition intermediates of Tn7 and retroviral-like transposons, respectively (Bainton *et al.*, 1991, Craigie, 2002, Sandmeyer *et al.*, 2002). Although generated by different mechanisms in each element, the overhangs will encounter the common fate of being removed during repair of the gaps. Besides a common structure shared by the strand transfer intermediates of Mu and retroviral elements, the transpososomes of these elements also share many structural features, including their extraordinary stability and a bent target conformation (Montano *et al.*, 2012, Maertens *et al.*, 2010). The bend positions a short target DNA segment within two transposase active sites, each of which holds one cleaved 3'OH end of the transposon; nucleophilic attack of the two DNA 3'OHs on target phosphodiester bonds spaced 5 bp apart completes the joining reaction of transposition. This chemistry is shared by all transposons, so it is likely that the target bend is also a shared by all transpososomes (Harshey, 2015). While Mu has an elaborate mechanism to disassemble the transpososome during transition to replication (Fig. S1), the results in this paper suggest that the replisome might participate in transpososome disassembly during non-replicative transposition, which is the most prevalent transposition mechanism. *In vitro* attempts to recapitulate the gap-filling reaction after retroviral transposition has identified several polymerases, ligase and FEN-1 nuclease, but the *in vivo* chromatin substrate is substantially different, and the participating enzymes are likely to be different (Daniel, 2006). Interestingly, like with Mu, DSB repair proteins have been implicated in repair of retroviral integration events (Daniel, 2006, Yang *et al.*, 2010). All these shared features hint at a common pathway of gap repair in all transposons.

Perspective

Mu has played a central role in the development of the mobile DNA element field (Harshey, 2012). Pioneering *in vitro* Mu experiments led to the unraveling of the phosphoryl transfer chemistry for all transposable elements (Mizuuchi & Baker, 2002). High-throughput integration assays modeled after Mu (Craigie *et al.*, 1991), led to the development and marketing of the HIV integrase inhibitor Raltegravir (Summa *et al.*, 2008). We expect our *in vivo* study on the repair of Mu insertions to provide equally important insights into the long-standing problem of how DNA transposons, retrotransposons, and retroviruses disassemble their highly stable transpososome intermediates and repair their insertions without duplicating themselves in the process, presenting perhaps a new target for drug development.

Beyond the repair of transposition events, our work has implications for understanding how polymerases stalled at DNA damage sites could signal removal of proteins that block repair of DNA lesions such as inter-strand crosslinks (Stingele *et al.*, 2014), or bypass the lesion entirely by re-initiating replication downstream of the damage (Ho & Scharer, 2010, McVey, 2010, Pathania *et al.*, 2011).

Experimental Procedures

Strains and growth conditions

All strains used in this work were derivatives of *E. coli* K-12 and listed in Table S1. They were propagated in LB media, except for *priA* and *polA* mutants, which were grown in 56/2 minimal medium: 0.06 M Na₂HPO₄, 0.04 M KH₂PO₄, 0.02% MgSO₄·7H₂O, 0.2% (NH₄)₂SO₄, 0.001% Ca(NO₃)₂, and 0.00005% FeSO₄·7H₂O, 0.2% glucose, with casamino acids at 50 µg/ml (Willetts *et al.*, 1969). P1 transduction was used to move mutations between strains (Miller, 1992).

Blocking replication in Dnats mutants

For all Dnats mutants, cells grown at 30°C were shifted to 42°C for variable times, depending on the mutation. DnaA_{ts} and DnaC_{ts} mutants were incubated for 90 min at 42°C to allow ongoing replication to terminate (Wechsler & Gross, 1971), while all other *ts* mutants were incubated for 30 min at 42°C, because these other mutants stop replication immediately at the non-permissive temperature (McMacken *et al.*, 1987).

Construction of pGam-GFP

Plasmids are listed in Table S2 and oligonucleotide primers in Table S3. The Gam-GFP fusion was amplified from the genome of strain SMR14334 using primers P1 and P2. The ~1.2 kb Gam-GFP product was digested by Sal I and Xba I and ligated into the same enzyme sites of the vector pRHA-113, where it was placed under the control of the rhamnose-inducible promoter. Similarly, a GFP-only control was amplified from same strain SMR14334 using primers P2 and P3. The resulting plasmids pGam-GFP and pGFP were verified by sequencing.

Construction of prophage Mu::TetO

The Mu lysogen strain HM8305 was used to construct prophage Mu::TetO (SJ012) as follows. First, *kan* was introduced next to the TetO array in pRS306X112TetO at the SalI-SpeI sites (using primers P4/P5) to give pTetO(*kan*). pMuHF was then constructed to introduce ~500 bp homology corresponding to either side of the SE (semi-essential) region (primers P6/P7 and P8/P9) in the prophage into which TetO_{kan} was to be substituted. Next, TetO_{kan} was cloned within the two arms of the SE region in pMuHF to give pTetO(*kan*)-MuHF. This plasmid was digested with SalI and recombined into the Mu prophage to produce a deletion of the SE (~3.6 kb; 4319 bp to 7953 bp on the Mu genome) concomitant with a substitution (~6kb) with TetO_{kan}, using λ-Red recombination (Datsenko & Wanner, 2000). The *kan* cassette was removed using Flp recombinase from pCP20. Finally, a non-essential region spanning ~2.4 kb at the right end of Mu (33,883 to 36,300 bp) was deleted by substitution with a *kan* cassette (primers P10 and P11), followed by removal of *kan* as

before. The latter deletion was required to maintain a genome length that could be packaged into viable phage.

Phage preparation

Wild-type Mu prophage inserted into *lacZ* gene in strain HM8305 carries a temperature sensitive (*ts*) allele of the lysogenic repressor and is induced by repressor inactivation at a high temperature as described (Au *et al.*, 2006). Phage from a 1 liter culture were precipitated with PEG 8000 and concentrated on a CsCl density gradient. Final concentration of purified phage was $\sim 10^{11}$ pfu/ml, estimated by plating on BW25113. Phage from the Mu::TetO lysogen were prepared in a smaller 50 ml culture and not concentrated on CsCl gradient. Instead, when cells reached an OD₆₀₀ of 0.4, they were concentrated by pelleting and resuspending in 10 ml of pre-warmed LB and induced as described above. Phage titers were $\sim 10^9$ pfu/ml.

Phage infection and purification of chromosomal DNA

200 μ l of overnight cultures were transferred into 20 ml LB media (5 mM CaCl₂ and 5 mM MgSO₄) and grown till an OD₆₀₀ of ~ 0.3 – 0.4 . All infections with wild type Mu phage were at MOI = 5 at either 30°C, 37°C or 42°C. Mu infections in the Dnats strains were carried out at 42°C. At various times after infection, total DNA was extracted, subjected to pulse field gel electrophoresis, and the gDNA band isolated as described (Au *et al.*, 2006).

Detecting integrated Mu and its flanking DNA in the *E. coli* genome by PCR

Mu and flanking DNA (FD) sequences inserted into the *E. coli* genome were amplified by standard PCR as described, and the products visualized on 1% agarose gels after staining with ethidium bromide (Choi & Harshey, 2010). Mu integration was detected by PCR using primers within the MuB gene, and FD DNA by primers that amplified the junction between right end of Mu and *lacZ* (Table S3). PCR was performed with 50–100 ng of template DNA, 10 pmol of primers, 10 μ mol of deoxynucleoside triphosphates, 2.5 units of Taq polymerase (Qiagen), 1x PCR buffer, and 1x Q solution in 25 μ l. The PCR conditions were 94°C for 2 min; 30–40 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 7 min as described. SS996 derived host strains include a partial *lacZ* gene, but this did not interfere with the assay because amplification was based on a second primer annealing inside Mu. Real-time or qPCR assays, were programmed in the ViiA7 sequence detector (Applied Biosystems) and the level of integrated Mu DNA was normalized to a chromosomal locus of 16S rRNA.

Iterative primer extension (IPE) assay

This assay has been described (Pato, 2004). One-directional PCR was performed with 1 μ g of total genomic DNA as a template, 5 pmol of a single primer which was 5'-end-labeled with γ -³²P, Taq 2X master mix solution (Promega). The primer was extended as follows: 94°C for 2 min; 60 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 20 s; and a final extension at 72 for 7 min. PCR products were separated on a 10% polyacrylamide sequencing gel and quantified with a phosphorimager, Typhoon 9500 (GE Life Science).

Flow cytometry

Flow cytometry was used to determine the number of chromosomal origins per cell. 1 ml culture samples were fixed by adding 9 ml of 95% ethanol and maintained at 4°C. These cultures were centrifuged at 6000g for 10 min in 4°C and the cell pellet was washed with and resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Cells were diluted to OD₆₀₀ of 0.1 and stained by addition of 5 µM SYTOX Green (Invitrogen) by incubating in the dark for 15 min. A total of ~20,000 viable cells were sorted in a BD Accuri™ flow cytometer, and the data were analyzed by FlowJo software.

Measuring the SOS response using GFP

This assay measures GFP expression from the promoter of the SOS-induced gene *sulA* (*PsulA-gfp*; (McCool *et al.*, 2004). Mu infections were carried out either in WT (SS996), or its *lexA3* (SS4294) and *lexA71* (SS4610) derivatives. *lexA3* is defective for SOS induction, while *lexA71* is constitutively induced. 30 min after Mu infection, cells were harvested and diluted 1:100 into TE buffer. Cells were sorted in the BD Accuri™ flow cytometer and analyzed as described above.

Visualizing Mu and DSBs *in vivo*

Mu::TetO location was visualized by expressing TetR-mCherry from pDB317 using 100 µM sodium salicylate. Double strand breaks (DSBs) were assayed by appearance of either Gam-GFP foci (Shee *et al.*, 2013) or RecA-GFP foci (Renzette *et al.*, 2005). Gam-GFP expression protocol was modified slightly from that of Shee *et al.* who expressed it for 4 hr prior to visualization (Shee *et al.*, 2013). Prolonged expression of Gam-GFP affects cell viability (Fig. S5). Therefore, expression was controlled by the tightly regulated rhamnose-inducible promoter in pGam-GFP by repressing it with 0.2% glucose until needed. 500 µM rhamnose was added only 30 min before Mu infection. RecA-GFP is expressed constitutively from its normal chromosomal location in the *recA-gfp* strain (SJ006).

Mu::TetO infection was carried out at an MOI of 1 in host strains already expressing TetR-mCherry. 15 min after infection, cells were placed onto 1% agarose pads at room temperature as described (Skinner *et al.*, 2013), and visualized within 5–10 min with an Olympus BX53 fluorescence microscope. Images were captured using cellSens standard software (version 1.6) from Olympus.

Western blot

After Mu infection for 30 min, 5×10^8 cells were harvested and lysed in lysis buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA and 0.02 % bromophenol blue). After boiling at 95°C for 5 min, samples were applied to 10% SDS-PAGE gel, transferred to a PVDF membrane (Bio-Rad) after electrophoresis, and the blot was probed with a polyclonal anti-MuB antibody (Parsons & Harshey, 1988), followed by HRP-conjugated goat anti-rabbit IgG (Bio-Rad), and detected using ECL western blotting analysis reagents (GE Healthcare) (Ausubel & *al.*, 2003).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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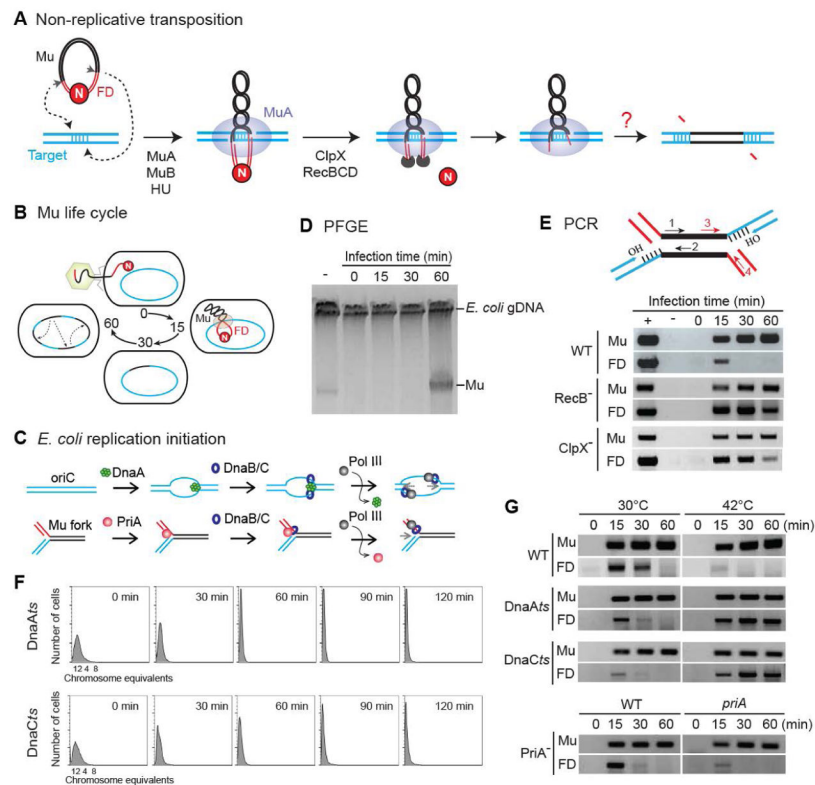


Fig. 1. Repair of Mu insertions is prevented if chromosome replication is prevented

(A) Known steps in the non-replicative (repair) pathway of Mu transposition. This pathway is used during integration of infecting Mu. The infecting genome is linear, and attached at both ends to long flanking DNA (FD) protected by Mu N protein. This DNA is variable in length (60 – 150 bp at the L end and 0.5 – 3 Kbp at the R end). MuN circularizes the DNA non-covalently, and protects it from nucleases. MuA catalyzes cleavage and strand transfer (integration) of Mu into the *E. coli* genome, assisted by MuB protein and host HU protein. The N protein is removed only after integration by an unknown mechanism assisted by the transpososome (purple ball), and the FD is degraded by RecBCD. Degradation is slowed in the absence of ClpX. *In vitro*, the final product of RecBCD degradation leaves 4 nt of the FD. This strand transfer intermediate with short flanks is likely the substrate for the final steps in repair, where the 5 bp target gaps are filled to generate a simple insertion. (B) Mu life cycle. After infection, Mu integrates into the *E. coli* genome, the FD is degraded, the Mu insertion is repaired, and Mu enters the lytic cycle. The approximate time (0–60 min) of these events is indicated. (C) Schematic of known mechanisms for replication initiation at *oriC* and during Restart of Mu replication in *E. coli*. (D) Preparation for the FD detection assay. At various times after infection, genomic DNA was subjected to pulse-field agarose gel electrophoresis (PFGE) to separate integrated Mu from free Mu, and the gDNA was excised for analysis by PCR. The (–) lane is an uninfected control where Mu DNA was added to the gDNA prior to electrophoresis, to assess contamination of the excised gDNA band with free Mu. Mu-length DNA at 60 min reflects packaged virions. (E) PCR assay for FD detection. Strains were infected with Mu at 37°C for the indicated times, and the isolated gDNA was tested by PCR to detect Mu or FD (*lacZ*) sequences using appropriate primers;

lacZ sequences linked to infecting Mu are not found in the host (Au *et al.*, 2006). + is Mu virion DNA and – is gDNA from the uninfected control lane in panel D. WT (BW25113); RecB⁻ (JW2788); ClpX⁻ (JW0428). (F) Monitoring inactivation of replication in DnaA*ts* (SS1424) and DnaC*ts* (SS1021) mutants by measuring chromosome equivalents. The mutants were held at 42°C for indicated times, without shaking, and fixed in ethanol before staining with the fluorescent DNA stain SYTOX Green. Stained cells were analyzed by flow cytometry as described under Experimental Procedures. By 60 min, there are no new rounds of replication in either mutant, as judged by the shift in the initial DNA content of ~2–4 chromosome equivalents to 1 chromosome equivalent. (G) FD removal depends on chromosome replication. DnaA*ts* and DnaC*ts* mutants were infected with Mu at both 30°C and 42°C. The latter infections were carried out after replication arrest for 90 min. In the WT (MG1655) infection at 42°C, FD is removed faster than at the lower temperatures. The PriA mutant (SS1448) and its WT parent (SS996) were infected at 30°C. Strains are listed and described in Table S1.

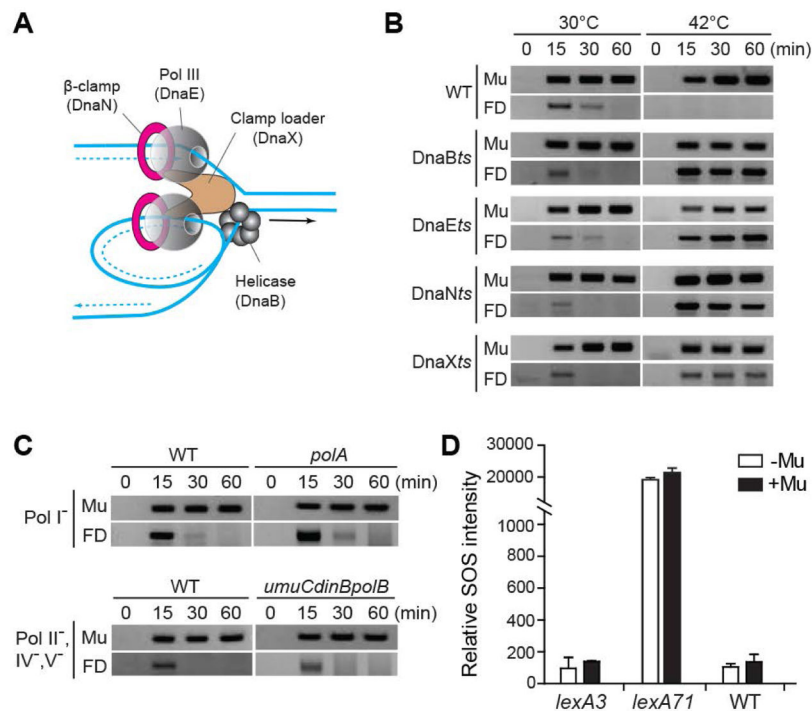


Fig. 2. The entire Pol III replisome machinery is necessary for FD removal: gap-filling polymerases are not required

(A) Replisome components tested in this experiment. Newly synthesized DNA is depicted by dotted lines. (B) Prior to Mu infection, replication was inactivated in the *ts* mutants by incubating cells at 42°C for 30 min. Mu and FD sequences were monitored as described in Fig. 1D and E. WT (SS996); *DnaBts* (SS6699); *DnaEts* (SS6239); *DnaNts* (SS6700); *DnaXts* (SS6698). (C) The *polA* (Pol I) mutant (SS2357) and its WT parent were infected with Mu at 30°C, while the triple *umuC dinB polB* (Pol II, IV, V) mutant (SS7346) and its WT parent were infected at 37°C, prior to analysis of Mu and FD. (D) SOS induction with or without Mu infection was monitored by expression of GFP under the control of the *sulA* promoter (*PsulA-gfp*) as described under Experimental Procedures (McCool *et al.*, 2004). Mu infections were carried out in *lexA3* (SS4294) and *lexA71* (SS4610) derivatives as well as in their WT parent, all carrying a chromosomal *sulA-gfp* fusion. *lexA3* is defective for SOS induction, while *lexA71* is constitutively induced.

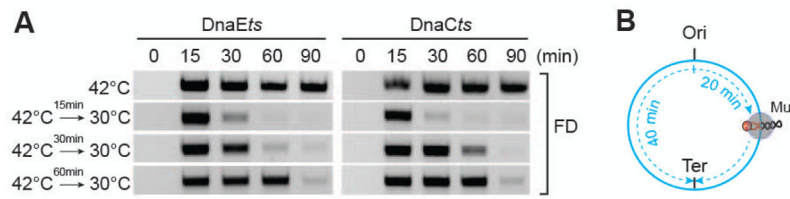


Fig. 3. Permitting blocked forks to resume replication, resumes Mu repair

(A) The indicated *ts* mutants were infected with Mu after inactivation of replication at 42°C for 30 min (DnaEts) or 90 min (DnaCts). Following infection (0 min), cells held at 42°C (top row) were shifted down to 30°C (15 – 60 min, rows 2–4) to release the replication block, and monitored for presence of the FD. (B) Schematic depicting the time taken by the bidirectional fork originating at *oriC* to reach *ter*. Mu integrates all over the chromosome. On average, the fork would encounter Mu in ~20 min.

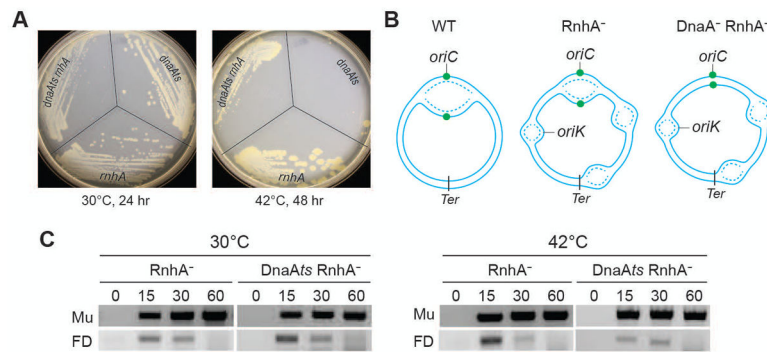


Figure 4. *oriC*-independent forks also promote FD repair

(A) Growth of the *rnhA* (N4704), *dnaA46* (*ts*; AU1054), and *rnhA dnaA46* double mutant (AU1066) strains on LB agar plates at 30°C and 42°C. The double mutant grows poorly even at 30°C. (B) Schematic depiction of replication originating at multiple ectopic locations in the RnhA mutant. (C) Mu integration and FD degradation in *rnhA* and *rnhA dnaA46* strains at 30°C and 42°C. The latter infections were carried out after replication arrest for 90 min. The WT parent for these strains is MG1655, shown in Fig. 1G.

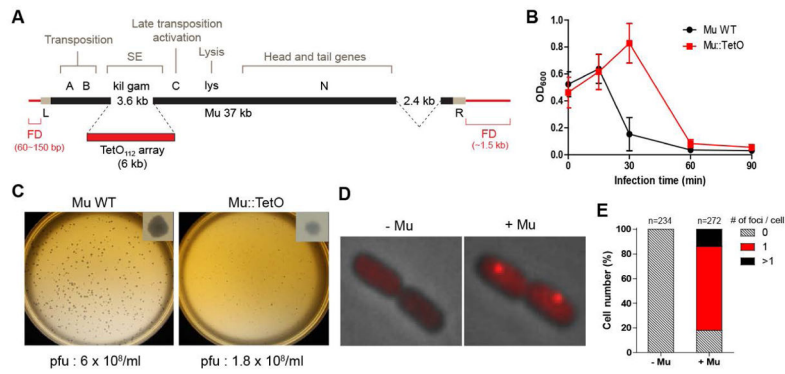


Figure 5.

Visualizing integrated Mu with TetR-mCherry.

(A) The TetO array was substituted for the SE region of the prophage, which is dispensable for phage growth. A compensatory deletion in another non-essential region near the R end restored the original Mu DNA length, ensuring that head-full packaging would yield viable phage progeny (Symonds *et al.*, 1987). See Experimental Procedures for construction details. (B) Lysis profiles of Mu::TetO (SJ012) and Mu wild-type (HM8305) prophage strains. Similar phage titers were obtained from both strains (see C). Error bars indicate standard deviation from the mean of triplicate data sets obtained from three independent colonies of the same strain. (C) Plaque morphologies of wild-type and Mu::TetO, titered on BW25113. Mutations in the SE region are known to affect plaque size (inset) (Symonds *et al.*, 1987). (D) Snapshot of Mu::TetO infection (MOI = 1) into WT strain BW25113 expressing TetR-mCherry (pDB317), photographed before (–Mu) and after ~15–20 min of infection (+Mu). (E) Quantitation of uninfected and infected cells in D by counting mCherry foci. > 80% of the cells had mCherry foci, and a majority of these had single foci.

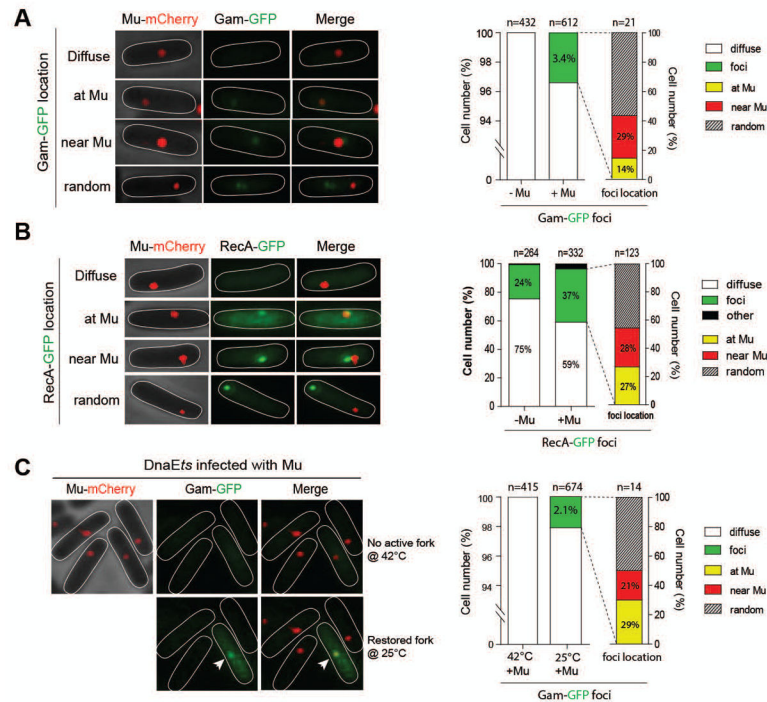


Fig. 6. Mu insertions generate replication-dependent DSBs in their vicinity

Mu::TetO is detected by TetR-mCherry (labeled Mu-mCherry) and DSBs by either Gam-GFP or RecA-GFP in WT (BW25113) or its DnaEts derivative (SJ005). (A) Left, snapshot of Mu-mCherry foci relative to Gam-GFP foci upon Mu infection of WT. Right, quantitation of the position of the green and red foci. GFP foci were scored as ‘at Mu’ when they completely overlapped with mCherry foci, ‘near Mu’ when at least their edges touched, and ‘random’ when they did not touch. Background fluorescence with no detectable foci was scored ‘diffuse’. (B) As in A, but with a RecA-GFP expressing strain. Foci with large or aberrant morphology are classified ‘other’. Using microbeTracker software (Sliusarenko *et al.*, 2011) the average area of a cell and of a fluorescent focus were calculated to be 1.78 μm^2 and 0.0675 μm^2 respectively, yielding a total of 26 independent sites that any focus can occupy in the cell. Two foci that touch occupy 4 times the area of one focus. The probability that any two foci will be stochastically near each other is then modeled by $4/26^2$ or slightly less than 0.6%, well below the experimentally observed 50%, which include foci that completely overlap. (C) Left, replication-arrested cells were infected with Mu and monitored for Mu-mCherry and Gam-GFP foci (top panel). The same frame of cells was photographed after releasing the replication block by shifting cells to 25°C (bottom panel). White arrow points to GFP focus that appeared after the temperature shift. Right, quantitation of foci after fork release, as in A. See also Fig. S6. See Experimental Procedures for details.

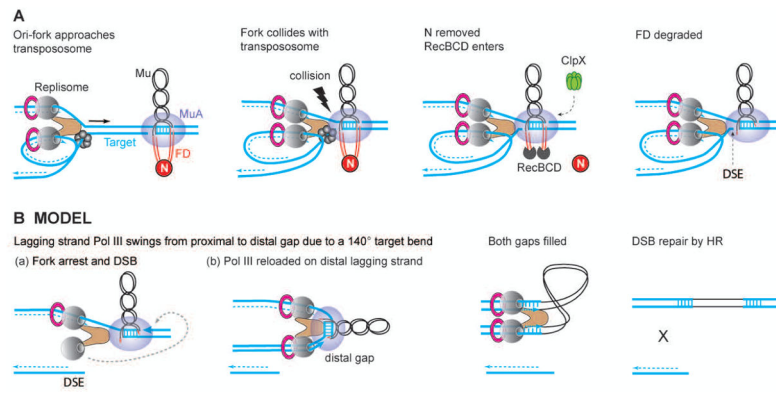


Fig. 7. Model depicting how the Mu transpososome first exploits incoming Pol III for just-in-time FD degradation, and next exploits Pol III stalled at the DSB for coordinated repair of both target gaps flanking Mu

(A) Sequence of repair events as deduced in this, and in prior work (Choi & Harshey, 2010, Choi *et al.*, 2014a). The Mu transpososome does not begin FD repair until the replication fork arrives. Interaction between the two complexes generates a ‘signal’ for N removal and RecBCD entry. The fork stalls because the transpososome is blocking its progress. (B) Scheme for how the DSB and the bent target is exploited for repair of both gaps during non-replicative Mu transposition. *E. coli* replisomes contain three polymerase molecules (Reyes-Lamothe *et al.*, 2010), but only two are shown for clarity. When the polymerase stalls at the transpososome, the Pol III subunit on the lagging strand (or the extra third subunit) reloads on the distal gap brought into proximity by the hairpin target bend within the transpososome (Montano *et al.*, 2012). As the polymerase moves forward, the transpososome is dislodged and both gaps are filled simultaneously. The DSE may not form until this step, but is shown earlier only to accommodate the target bend. A nuclease must trim the 4 nt flanks, and a ligase seal the remaining nicks. The DSE is repaired by homologous recombination (HR).