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Requirement of IS*911* replication before integration defines a new bacterial transposition pathway

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Movement of transposable elements is often accompanied by replication to ensure their proliferation. Replication is associated with both major classes of transposition mechanisms: cut-and-paste and cointegrate formation (paste-and-copy). Cut-and-paste transposition is often activated by replication of the transposon, while in cointegrate formation replication completes integration. We describe a novel transposition mechanism used by insertion sequence IS*911*, which we call copy-and-paste. IS*911* transposes using a circular intermediate (circle), which then integrates into a target. We demonstrate that this is derived from a branched intermediate (figure-eight) in which both ends are joined by a single-strand bridge after a first-strand transfer. *In vivo* labelling experiments show that the process of circle formation is replicative. The results indicate that the replication pathway not only produces circles from figure-eight but also regenerates the transposon donor plasmid. To confirm the replicative mechanism, we have also used the *Escherichia coli* terminators (*terC*) which, when bound by the Tus protein, inhibit replication forks in a polarised manner. Finally, we demonstrate that the primase DnaG is essential, implicating a host-specific replication pathway.

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Introduction

To be successful, transposable elements must proliferate through populations but exhibit controlled activity to avoid damaging their host genomes. The factors involved in their proliferation are not well characterised. Simply maintaining transposon copy number by passive replication would appear to be a poor strategy for promoting transposon spread within and between genomes. A more efficient strategy would be to couple transposition with replication of the element, and indeed a replication event is associated with movement of many transposons within the cell.

This can be achieved in several ways. One, adopted by the bacterial insertion sequence IS*10*, is to activate transposition following its passive replication by passage of a host replication fork during a normal host cycle. Activation depends on Dam methylation sites in the ends of IS*10*, which become transiently hemimethylated following passage of the fork. This state renders the ends more active in transposition and activates the Tpase promoter located in one end, resulting in increased Tpase synthesis (Roberts *et al*, 1985). Transposition is therefore effectively preceded by duplication of the element. In this case, if one of the two daughter ISs undergoes transposition, it is not difficult to imagine how the cleaved donor chromosome branch from which the IS has been excised might undergo repair by a copy-choice pathway using the intact sister copy. This would replace the excised IS, therefore effectively increasing its copy number.

A second strategy is to assure that precise replication of the transposon occurs as an integral part of the transposition reaction itself. This strategy has been adopted by bacteriophage Mu (Chaconas and Harshey, 2002), the Tn*3* family of transposons (Sherratt, 1989; Grindley, 2002), certain IS elements such as members of the IS*6* family (Mahillon and Chandler, 1998) and in certain circumstances with IS*1* (Turlan and Chandler, 1995). For these, only one of the transposon DNA strands is cleaved at each end and transferred, leaving the transposon covalently attached to both donor and target replicons. In the case of phage Mu, a specific set of host replication enzymes is assembled at the fork created by the initial strand transfer from the transposon to the target DNA (Kruklitis and Nakai, 1994; Nakai and Kruklitis, 1995). Replication circumvents the need to resolve the second transposon strand. It generates an intermediate in which one end of each transposon is attached to the donor DNA and the other end to the target. The resulting cointegrate structure carries two copies of the transposon. Donor and target molecules are joined with a single transposon copy in a direct orientation at each donor–target junction. This intimate relationship between transposon and host is highlighted by the observation that, under conditions in which they fail to undergo their normal excisive transposition cycle due to Tpase mutation (e.g. Tn*7*; May and Craig, 1996) or mutation in the ends (e.g. IS*903*; Tavakoli *et al*, 1997; Tavakoli and Derbyshire, 2001), these elements are processed by the host replication machinery to generate cointegrates.

We address here the role of replication in the transposition of IS*911*, a member of a widespread group of transposable elements, the IS*3* family of bacterial insertion sequences.

During the first step of IS*911* transposition, a single DNA strand of one end is cleaved and transferred to the same strand 3 bases from the opposite end (Figure 1; see Rousseau *et al*, 2002) to generate a single-strand bridge between the

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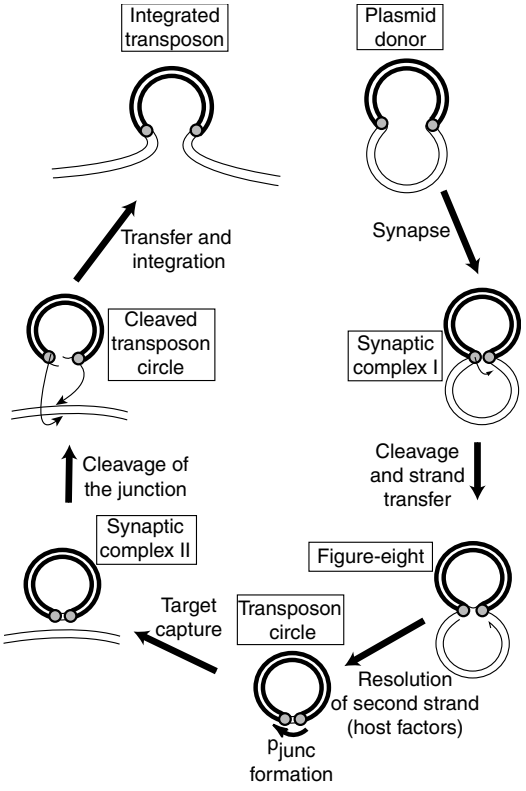


Figure 1 IS*911* transposition pathway. The transposon (heavy line), plasmid backbone (lighter line), and transposon termini (grey circles) are indicated. The transposon ends are brought together in a synapse (synaptic complex I). A first single-strand cleavage occurs initially at one end of the IS, catalysed by the low level of Tpase expressed from a weak promoter, pIRL, present in IRL. The free 3′-OH generated is then directed to attack the opposite end on the same DNA strand (strand transfer) to generate a figure-eight form in which both ends are joined by a single-strand bridge, leaving an unjoined DNA strand with a free 3′-OH (half arrow) on the vector plasmid. The figure-eight is converted into a transposon circle where the formation of a strong promoter, p_{junc}, induces high-level synthesis of transposon proteins (Ton-Hoang *et al*, 1997; Duval-Valentin *et al*, 2001). A second Tpase-mediated synaptic complex (synaptic complex II) is formed upon target capture. The Tpase then undergoes the final steps of cleavage and strand transfer into the target DNA (integration). Insertion results in the disassembly of the strong promoter and returns to a low level of transposition activity.

two transposon ends. If this is located on a circular plasmid, the molecules assume a figure-eight structure, where the IS*911* terminal inverted repeats (IRL and IRR) are separated by three bases derived from external flanking DNA. If supplied with high levels of Tpase (OrfAB) *in vivo*, figure-eight molecules are found together with free circularised transposons (circles) in which the ends are abutted. These transposon circles are intermediates in IS*911* transposition and have been shown to be highly competent for integration into a DNA target *in vivo* (Ton-Hoang *et al*, 1997) and *in vitro* (Ton-Hoang *et al*, 1998). Preliminary kinetic studies, in which chloramphenicol was used to stop Tpase synthesis, suggested that the figure-eight forms were precursors of the transposon circles (Polard and Chandler, 1995). However, *in vitro*, enriched preparations of OrfAB generate figure-eight molecules but not transposon circles (Polard *et al*, 1996). This observation raised the possibility that transposon circle formation required host factors not present in the *in vitro* reactions. This notion was reinforced by the observation that circularised transposons could be detected following introduction of preformed figure-eight molecules into naïve cells in the absence of Tpase (Turlan *et al*, 2000).

Here we describe a series of kinetic experiments, which clearly show that figure-eight molecules are precursors of transposon circles and that the host replication machinery is involved in this step. Figure-eight molecules appear before transposon circles and disappear with a concomitant increase in transposon circles. *In vivo* labelling experiments using ^{3}H-thymidine and plasmids with a temperature-sensitive replication apparatus showed that the process does not require replication initiated from the plasmid replication origin, but that it does require active Tpase presumably to generate figure-eight molecules. The results also suggest that the replication pathway not only produces circles from figure-eight molecules, but also regenerated the transposon donor molecule, implying that both strands are replicated. We have used the *Escherichia coli* replication terminator sequences, *terC*, which, if bound by the host Tus protein, inhibit replication forks in a polarised manner. When introduced within the transposon in an appropriate orientation, *terC* was found to inhibit figure-eight resolution to circles in the presence of Tus, but not in its absence. Finally, we have initiated studies to determine which host enzymes are involved in the replicative formation of circles from figure-eight forms. Present experiments demonstrate that the primase DnaG is essential. Transposition of IS*911* can therefore be considered to occur by a copy-and-paste mechanism (Curcio and Derbyshire, 2003).

Results

Figure-eight molecules are precursors of transposon circles

In the model of the IS*911* cycle (Figure 1), figure-eight intermediates were proposed to form before and to give rise to transposon circles. To confirm this, we have used an *in vivo* kinetic approach to determine the chronology of appearance of the two types of molecule. For this, we have exploited the observation that the activity of IS*911* Tpase is temperature sensitive (Haren *et al*, 1997).

The kinetics of figure-eight and circle formation were determined using pLH39, a p15A-based plasmid carrying an artificial IS*911*-derived transposon and an *orfAB* gene controlled independently by the p_{lacuv5} promoter and located outside the element (Figure 2). Cultures were grown at 42°C to eliminate residual activity of the naturally temperature-sensitive OrfAB Tpase. This prevents accumulation of intermediates before Tpase induction. The culture was transferred into LB medium, pre-warmed at 30°C, and production of Tpase was induced. Samples were withdrawn at different times and plasmid DNA was isolated, digested with *Bam*HI, which cleaves once within the transposon and once within the donor plasmid backbone. The enzyme therefore generates two fragments from the donor plasmid, linearises transposon circles, and converts the figure-eight structures into χ forms which migrate high in an agarose gel (Polard and Chandler, 1995). The results shown in Figure 2 revealed that figure-eight molecules arose before 5 min of induction, whereas transposon circles were visible after only 12–16 min. This

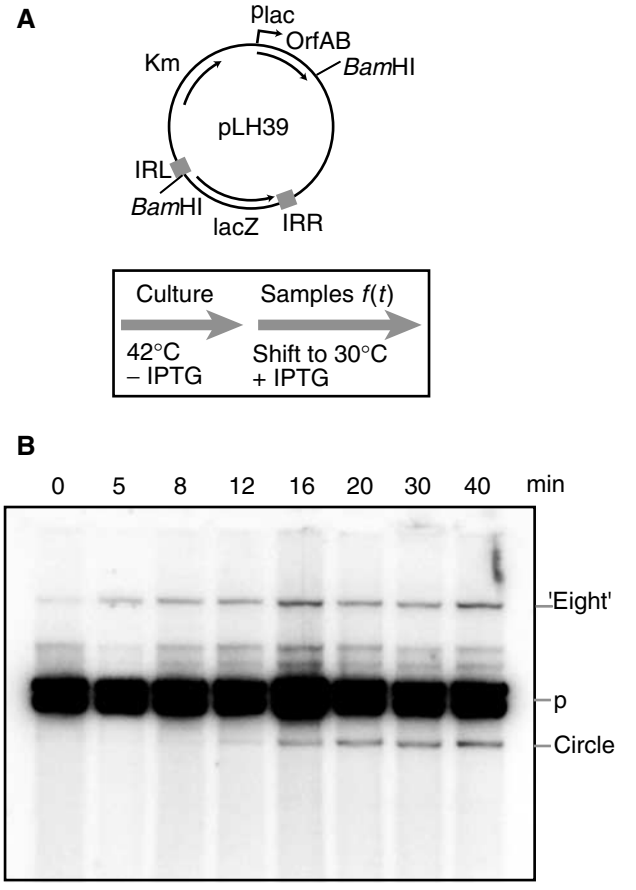


Figure 2 *In vivo* kinetics of figure-eight and circle formation. (**A**) Plasmid structure and experimental regime. Plasmid pLH39 is a p15A-based plasmid carrying an artificial IS*911*-derived transposon and an independent OrfAB gene under control of p_{lacuv5}. The position of *Bam*HI sites to separate different molecules is shown. The culture was grown at 42°C (OrfAB activity is temperature sensitive) to ensure that there was no residual Tpase activity. At $t = 0$ ($OD_{600} = 0.6$), the population was transferred into LB medium prewarmed at 30°C and production of Tpase was induced with IPTG. Samples were withdrawn at different times, growth was stopped by addition of an sodium azide/ice mixture, and cleared lysates were then made. (**B**) Separation of plasmid species. Agarose gel of *Bam*HI-digested DNA samples removed at times 0–40 min (shown above the lanes). The separated species were visualised by hybridisation directly in the dried gel with a transposon-specific oligonucleotide probe. The position of the χ forms generated by *Bam*HI digestion of the figure-eight molecules is shown ('eight'), together with the two bands obtained from the donor plasmid (p) and the *Bam*HI-linearised transposon circles. Note that some additional minor bands are due to incomplete digestion of plasmid molecules.

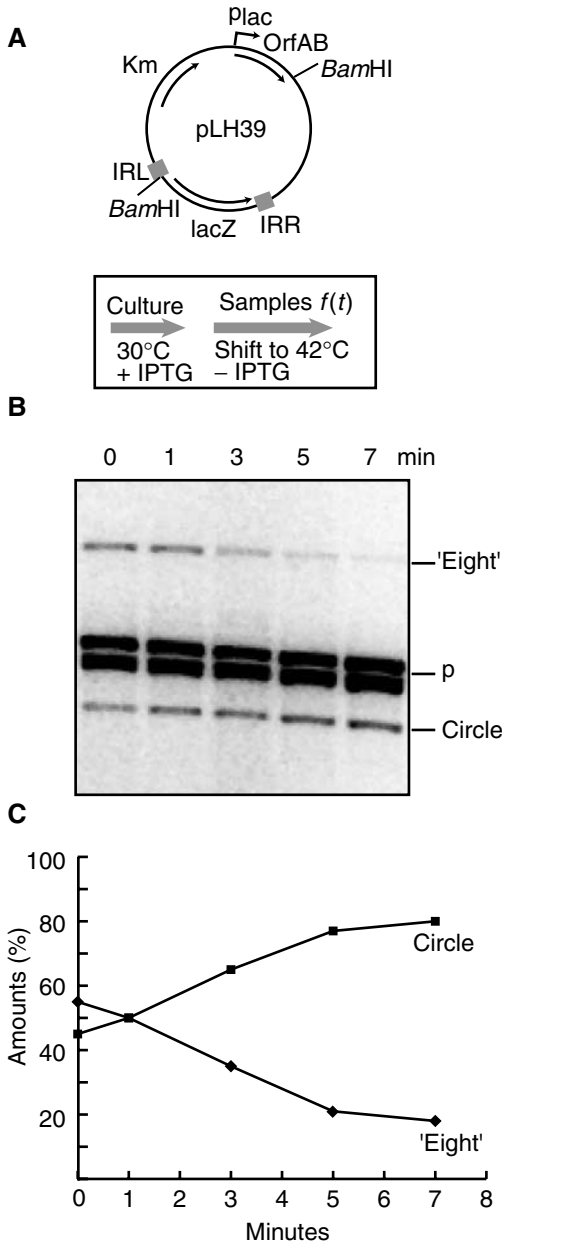


Figure 3 *In vivo* kinetics of figure-eights to circles conversion. (**A**) Plasmid structure and experimental regime. The plasmid used was the same as described in Figure 2 (A). The culture was grown at 30°C in the presence of IPTG to induce Tpase synthesis and accumulate figure-eight. At $t = 0$, the culture was transferred into fresh LB medium prewarmed at 42°C without IPTG to inactivate Tpase and stop the induction. Samples were withdrawn at different times and treated as described in Figure 2. (**B**) Separation of plasmid species. Agarose gel showing details of samples taken between 0 and 7 min. The separated species were visualised by hybridisation as in Figure 2. The symbols are as described in Figure 2. (**C**) Quantitation of gel shown in (B). The values shown were calculated as follows: the intensities of figure-eight and circle bands were measured and the sum was used to define a value of 100%. The level of parental plasmid was used to normalise sampling variations from well to well.

suggests that figure-eight molecules are generated before transposon circles.

To determine if the figure-eight molecules are precursors of transposon circles, the experimental regime was inversed: cultures of pLH39-carrying cells were grown at 30°C in the presence of 0.1 mM IPTG to induce Tpase expression and accumulate figure-eight and circle intermediates. The culture was centrifuged and the cells transferred into fresh LB medium pre-warmed at 42°C without IPTG (Figure 3A). This inactivates any residual Tpase activity and prevents further expression. Samples were withdrawn at different times and treated as described above. This experiment showed (Figure 3B) that figure-eight forms disappeared, while levels of transposon circle increased concomitantly. Quantitation of the amounts of each species (Materials and methods) showed that the reduction in figure-eight molecules

was mirrored by a similar increase in the transposon circle species (Figure 3C). This strongly suggests that figure-eight molecules are precursors of transposon circles and, since Tpase is largely inactivated under these growth conditions, it suggests that host factors are responsible for this conversion.

OrfAB-dependent DNA synthesis

Previous studies had shown that transposon circles could be detected when pre-formed and deproteinised figure-eight molecules were introduced into naïve cells which do not contain IS*911* proteins (Turlan *et al*, 2000). This implied that host factors are involved in this step of circle formation. Thus, transposon circles might be generated from figure-eight molecules either by a recombination process, which would effectively excise the transposon as a circle from the donor plasmid, or by replication of the transposon, which would lead to circle production and regenerate the parental donor plasmid (Figure 4; Polard *et al*, 1996). In the experiments described above, the absence of detectable levels of appropriate DNA fragments representing the donor backbone lacking the transposon suggested that transposon circles are not formed by simple excision from the parental donor plasmid (Figure 4C). Alternatively, transposon circle formation could involve replication. This could use either the plasmid origin of replication (Figure 4A) or the 3′-OH generated in the donor backbone by the initial strand transfer event, which generated the figure-eight single strand bridge (Figure 4B).

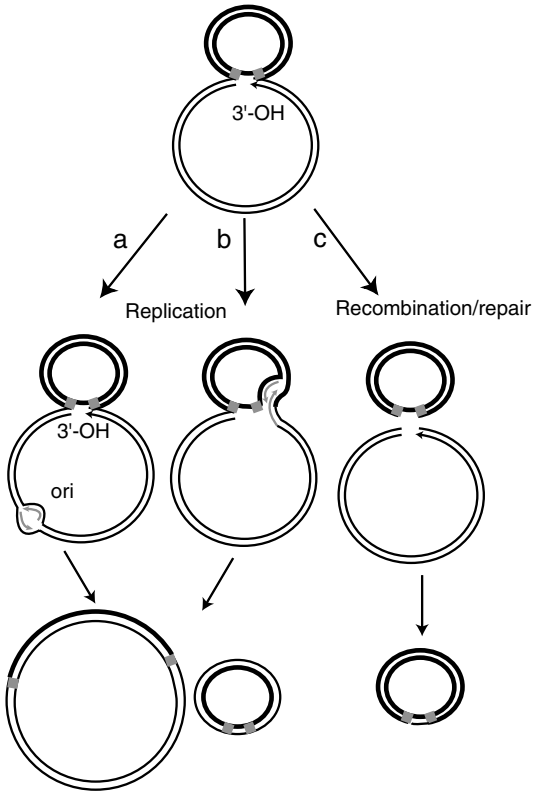


Figure 4 Model of three possible pathways for resolving figure-eight molecules to circles. The figure-eight molecule carrying a 3′-OH is shown above. The two left-hand drawings show circle formation driven by replication from the plasmid origin of replication (left) or from the Tpase-generated 3′-OH (centre). In both cases, replication would generate a double-stranded transposon circle and regenerate the original parental plasmid. Conversion using a recombination/repair pathway is shown on the right. Host-mediated repair would be expected to lead to either destruction or recircularisation of the donor plasmid backbone.

To test the replicative model and to distinguish between these possibilities we investigated circle formation using a temperature sensitive replication mutant of the low copy number plasmid pSC101, pPR4, carrying an artificial IS*911*-based transposon and a compatible p15A-derived plasmid, pAPT111, which carries the OrfAB gene under control of the p_{lac} promoter or pAPT110 without the Tpase gene (Figure 5A).

Cultures of a *thyA* strain carrying both plasmids were grown at 30°C in minimal medium complemented with thymine (Materials and Methods). OrfAB synthesis was induced and growth was continued to accumulate figure-eight molecules. The culture was transferred into fresh prewarmed medium without thymine or IPTG at 45°C to inactivate residual OrfAB activity, to prevent further production and to block replication of the temperature sensitive pPR4 donor plasmid. The culture was pulse-labelled with ^{3}H-thymidine. Alkaline lysates were prepared and plasmid DNA was digested with *Eco*RV (which cleaves twice in pPR4, once within the transposon circle and once in pAPT111) and analysed by agarose gel electrophoresis. Note that this lysis procedure removes a majority of the figure-eight molecules since they contain a single strand break and are irreversibly denatured. In the absence of OrfAB (pAPT110, Figure 5, lane 1) only the p15A derivative was labelled. No labelling of the pSC101 derivative was observed demonstrating that no detectable vegetative replication of pPR4 occurred under these conditions. However, in the presence of OrfAB (lane 2), the Tpase donor plasmid, pAPT111, the two transposon donor plasmid DNA fragments and the linearised transposon circle were labelled. Thus under conditions in which replication from the plasmid origin is inhibited, DNA synthesis requires the presence of OrfAB. This presumably occurs using the free 3′-OH generated in the donor backbone (Figure 1) as a primer for replication.

Moreover, since both transposon circles and the transposon donor plasmid were labelled, this suggests that parental transposon bearing plasmids can be regenerated from figure-eight forms concomitantly with circle formation.

terC affects figure-eight resolution in a polar manner

To further examine the replicative nature of transposon circle formation we made use of the host *ter*/Tus system. This is composed of short polarised DNA sequences, *ter*, which bind the *E. coli* Tus protein in an oriented manner and delay passage of a replication fork (Hill *et al*, 1988; Kuempel *et al*, 1989). It seemed possible that placing a *ter* site within the transposon may affect replicative formation of transposon circles in the presence of Tus but not in its absence whereas placing the site in the donor plasmid backbone outside the transposon should have no effect on transposon circles (Figure 6).

One difficulty with this approach is that the initiating strand transfer event which generates a figure-eight molecule is not polarised. It can occur using either the right or left end and therefore gives rise to a mixed population of figure-eight molecules. Thus replication, to generate transposon circles, would be expected to occur from either end in the mixed

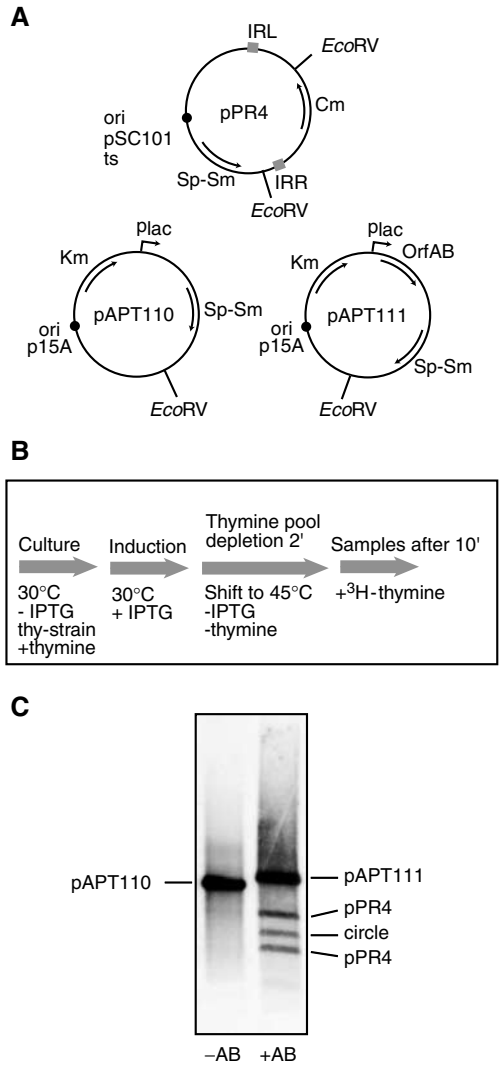


Figure 5 ^{3}H-thymidine incorporation *in vivo* during circle formation. (**A**) Plasmid structure. The temperature-sensitive plasmid replication mutant, pPR4, carrying the IS*911*-based transposon with a chloramphenicol resistance gene, Cm, located between correctly oriented IS*911* ends (IRL and IRR), is shown together with both the p15A-derived Tpase vector plasmid, pAPT111, carrying the *orfAB* gene, and its isogenic parent, pAPT110, lacking this gene. (**B**) Experimental regime. A culture of a *thyA*$^{-}$ strain carrying a temperature-sensitive pSC101 derivative as a transposon vector (pPR4) and either pAPT110 or pAPT111 was grown at 30°C in minimal medium complemented with thymine, and OrfAB synthesis was induced with IPTG. After growth to $OD_{600} = 0.6$, the culture was transferred into fresh medium without thymine or IPTG at 45°C in order to rapidly inactivate OrfAB production and replication of the transposon-carrying plasmid. After 2 min of incubation to deplete the internal pool of thymine, labelling with ^{3}H-thymidine was performed for 10 min, alkaline lysates were prepared, plasmid DNA was digested with *Eco*RV and analysed by agarose gel electrophoresis. (**C**) Agarose gel electrophoresis. The species were visualised directly by radio-luminescence as described in Materials and methods. Left lane: control experiment in the absence of OrfAB. Only the p15A-based vector plasmid pAPT110 is labelled, confirming that the temperature-sensitive pPR4 is not replicated at the nonpermissive temperature and that no circles were detected. Right lane: both the OrfAB-carrying p15A-based vector plasmid and the temperature-sensitive pPR4 plasmid with the transposon are labelled.

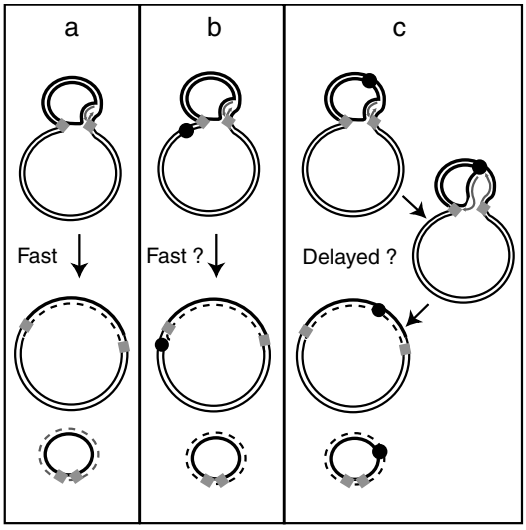


Figure 6 Scheme showing experiments to confirm the replicative pathway of figure-eight resolution in the presence of different locations of *terC* sites. *ter* sites and the Tus protein inhibit replication fork movement in a polar manner. *terC* sequences were therefore introduced either outside (scheme b) or inside (scheme c) an IS*911*-derived transposon in both orientations. The kinetics of circle formation would be expected to be affected differentially, depending on the orientation of *ter*. The predicted effects on the resolution rate are proposed. The newly replicated strand is shown as a dotted line.

figure-eight population. In order to orient the first strand transfer, we used IS*911* derivatives which carried a mutation of the first two base pairs of IRR or of IRL. These mutations inhibit the ability of the mutated end to undergo strand transfer but do not affect its ability to act as a target end (Polard and Chandler, 1995). The *ter* sites were introduced into plasmids carrying these IS*911* variants (IRL* with IRR, or IRL with IRR*). Plasmids (Table I) were constructed with *terC* sites inside the IS*911*-based transposon in either orientation (pDV41, 42, 43 with IRL*, or pDV48, 49, 50 with IRR*) or in the donor plasmid backbone (pDV45 and 46). Plasmids pDV43 and pDV50 harbour two *terC* sites in tandem (Figure 7 and Table I).

The appearance of transposon circles was analysed for all plasmids in both *tus*$^{+}$ or *tus*$^{-}$ strains. Strains were grown at 42°C and then shifted to 30°C in the presence of IPTG. Since the effect of *ter*/Tus is to delay a replication fork rather than to completely block it, several induction periods were tested. Plasmid DNA was extracted, digested with *Eco*RV and analysed as for the experiments shown in Figure 5. The clearest results were obtained after 30 min of induction and are shown in Figure 7.

TerC located in either orientation in the donor backbone (pDV45 and pDV46) showed no effect compared to the parental plasmid (pDV44) in the *tus*$^{+}$ strain (Figure 7A). However, clear effects were observed when the site was located within the transposon. In the case of the IRL*-carrying plasmids, transposon circles appeared to be reduced in pDV42 but not when the *terC* site occurred in the opposite orientation (pDV41 and pDV43) (Figure 7B). However, in the IRR* plasmids this pattern was inversed: plasmid pDV49 (equivalent to pDV42) continued to produce transposon circles while plasmid pDV48 and pDV50 did not (Figure 7C). Note that the *Eco*RV restriction site within the donor

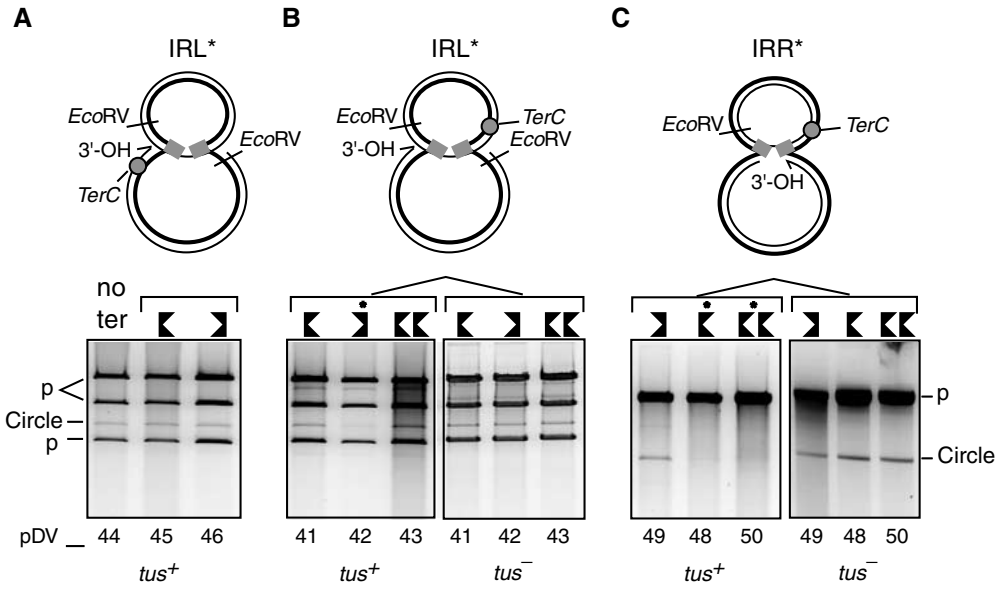


Figure 7 *terC* affects figure-eight resolution in a polar manner. IR donor mutants were used to obtain unique figure-eight populations and to orient the potential replication fork (**A**–**C**, top). The 1% agarose gels were stained with Sybr green. IRL* or IRR* donor mutants (A–C, bottom) were tested separately with different positions (shown as grey circles, top) and orientations of *terC* sites (shown by oriented black symbols, bottom; Table I). These were placed outside the transposon (pDV45 and 46 with IRL*; A) or inside the transposon with IRL* (pDV41, 42, 43; B) or with IRR* (pDV48, 49, 50; C). pDV43 and 50 harbour two *terC* sites in tandem. The right-hand panels (B, C) show the results obtained in a *tus*$^-$ strain. The major upper band marked p in (A, B) represents linearised pAPT111. The lower two bands marked p (A, B) represent two fragments generated from the transposon-carrying plasmids. Introduction of IRR* (C) resulting in the elimination of one of the two *Eco*RV sites from the parental plasmid pRP4 (Figure 5). These plasmids are therefore linearised by *Eco*RV and migrate at the same position as the linearised Tpase donor plasmid. The band corresponding to plasmid circles is also indicated. Additional minor bands migrate at positions consistent with partially digested products. Lanes in which transposon circle formation was delayed are indicated (*).

backbone was removed during the construction of these plasmids and the transposon and Tpase donor plasmids migrate as linear molecules at the same position in this gel. No inhibition was detected with any of the plasmids in the *tus*$^-$ control strain (Figure 7B and C, right panels).

Thus in the presence of Tus, *terC* exhibited permissive and nonpermissive orientations depending on the polarity of strand transfer. Furthermore, the nonpermissive orientation was that expected for replication from the free 3′-OH on the donor backbone. This result confirms that circle formation uses a host-specific replicative pathway.

Disappearance of figure-eight forms is delayed in a dnaG$_{ts}$ mutant

In a preliminary attempt to define the host factors necessary for replicative circle formation, we tested the effect of a mutation in the *dnaG* primase. A temperature sensitive *dnaGts* mutant gave a clear effect. Figure-eight molecules were generated from pLH39 in cultures grown at 30°C as described above (Figure 3). Following a shift to 42°C and elimination of IPTG, samples were removed, cleared lysates were prepared and digested with *Bam*HI and the resulting plasmid DNA species were separated by agarose gel electrophoresis. The results (Figure 8B) showed that the disappearance of the figure eight form was greatly delayed in the *dnaGts* strain compared to the wild-type strain.

Figure-eight molecules had disappeared by between 6–9 min in the wildtype strain whereas they persisted for 30–40 min in the *dnaG* mutant. In the latter case, figure-eight molecules disappeared abruptly after 30 min. This may be the result of degradation or of rescue by another mechanism (see Discussion).

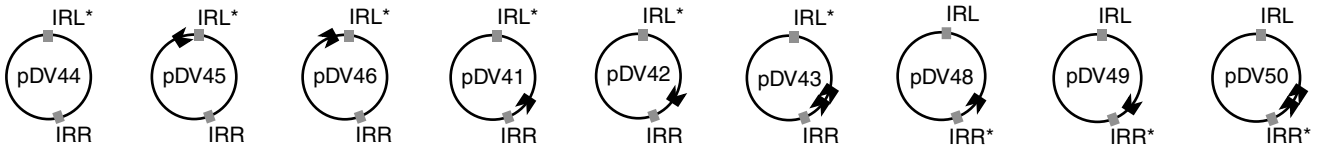
These results therefore suggest a fundamental role for the DnaG primase in conversion of figure-eights to circles, and thus strongly support a replicative model for circle formation.

Discussion

The strategy adopted by IS*911* and probably other IS*3* family members differs notably from other known transposition mechanisms. A distinguishing feature lies in the first step of strand cleavage and transfer in which only one strand at one IS end is cleaved to generate a 3′-OH. This then attacks the same strand 3 bp into the DNA flanking the opposite end to generate a single-strand bridge between the abutted ends. Although asymmetric, either end can serve at the attacking end. This is to be contrasted with other transposition processes in which both ends are cleaved simultaneously and are either transferred to the target without cleavage of the second transposon strand (e.g. Tn*3* (Grindley, 2002), Mu (Chaconas and Harshey, 2002), and members of IS*6* family (Chandler and Mahillon, 2002)) or following second strand cleavage and excision of the element (e.g. Tn*7* (Craig, 1996), IS*10* (Kleckner *et al*, 1996), Tn5, (Reznikoff, 2002)).

Another particularity of IS*911* transposition is that covalently closed transposon circles are generated as transposition intermediates (see (Rousseau *et al*, 2002)). This is also the case for IS*2*, IS*3* and IS*150* (Lewis and Grindley, 1997; Sekine

Table I



Constructions showing IRs mutated for donor activity and position and orientation of *terC* sites (◤) Permissive (P) or Non-Permissive (NP) orientations of *terC* terminators.

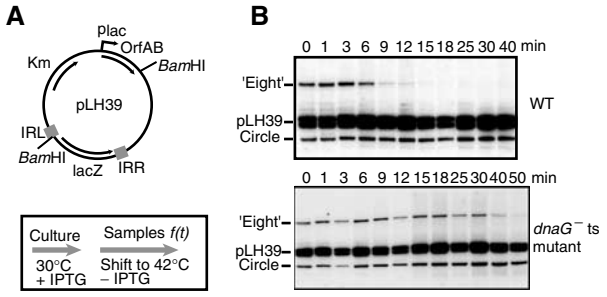


Figure 8 *In vivo* kinetics of figure-eight to circle conversion in a *dnaGts* strain. (**A**) Plasmid structure and experimental regime. After production of figure-eight molecules at 30°C (Tpase OrfAB and DnaG active (A)), the conversion of figure-eights to circles of a wild-type strain was compared with the *dnaG*ts strain at 42°C. Samples were withdrawn at different times and treated as described in Figure 2. (**B**) Separation of plasmid species by agarose gel electrophoresis. The top panel shows results obtained with the wild-type strain, while the lower panel presents the results obtained with the *dnaG* mutant. Numbers above the gel indicate the time in minutes after the temperature shift.

et al, 1999; Haas and Rak, 2002). The experiments presented here provide evidence that circular IS*911* transposition intermediates are derived from the bridged figure-eight molecules by a replicative mechanism. Kinetic experiments demonstrated that the figure-eight forms appear first, while transposon circles are produced only after a lag of about 10 min (Figure 2). Moreover, in chase experiments, preformed figure-eight molecules disappeared and transposon circles accumulated with identical kinetics and in similar quantities (Figure 3). No species with the migration properties expected of the replicative donor backbone alone (deleted for the transposon) were observed either here or in previous experiments (see for example Polard and Chandler, 1995). This suggests that simple excision of the transposon does not occur. These results extend those obtained previously (Polard and Chandler, 1995) which used a more aggressive treatment, addition of chloramphenicol, to inhibit total protein synthesis.

Experiments using plasmids in which replication initiation is temperature sensitive (pSC101ts) and which carried an artificial IS*911*-based transposon indicated that both transposon circles and the donor plasmid with the transposon were rapidly labelled by ^{3}H-thymidine *in vivo* in the absence of plasmid replication but after prior expression of the OrfAB Tpase. No labelling was observed in the absence of OrfAB (Figure 5). Concomitant appearance of ^{3}H-labelled circles and transposon-bearing plasmids in conditions where the initiation of replication of the transposon donor plasmid was blocked is consistent with replication of both DNA strands. The absolute dependence on the prior presence of the OrfAB and therefore presumably of figure-eight intermediates is consistent with initiation of replication from the figure eight junction. The results therefore support the replicative model shown in Figure 4B.

This type of model was further supported by the observation that programmed IS-replication is specifically affected by the presence of the replication terminator *terC*. These sites are normally located around the terminus of chromosome replication in a region diametrically opposite to the replication origin (Hill *et al*, 1988; Mohanty *et al*, 1996; Bussiere and Bastia, 1999). Interaction between *ter* sites and the Tus protein inhibits replication fork progression in an orientation specific manner (Khatri *et al*, 1989; Lee *et al*, 1989; Mulugu *et al*, 2001). This property was used to test the effect of *ter* sites introduced within the transposon or within the donor plasmid backbone in both permissive and nonpermissive orientations for oncoming replication forks. However, IS*911* can use either IRL or IRR to perform the first strand cleavage and transfer. This produces two different types of figure-eight molecules in the population with the resulting free 3′-OH on one or other strand (see Figure 1) and would result in a mixed population in which a given figure-eight molecule formed using either the left or right end (Figure 7, top). This situation complicates the use of polarised *ter* sites. Potential forks were therefore oriented by mutation of the terminal 5′-CA-3′dinucleotide of IRL (Figure 7A and B) or of IRR (Figure 7C). The dinucleotide is essential for the first cleavage and strand transfer (Polard and Chandler, 1995). Such mutants generate figure-eight molecules of unique polarity. The results clearly showed that circle formation was transiently paused only if *ter* sites were located within the transposon and in a specific orientation. In each case, the orientation was that expected to counteract replication forks initiated from the 3′-OH on the donor plasmid backbone generated by strand transfer. The observed delay in appearance of transposon circles was due to the specific action of the *ter* sites since it did not occur in a *tus*$^{-}$ strain (Figure 7).

While these results demonstrate that formation of transposon circles is replicative, they do not reveal the nature of the host enzymes involved. We examined the effects of the DnaG primase in figure-eight to circle conversion. Using a *dnaG*$_{ts}$ strain the disappearance of figure-eight molecules was observed to be significantly delayed at the nonpermissive temperature compared to the wild-type strain (Figure 8). Since DnaG is the host primase necessary for synthesis of Okazaki fragment primer RNA involved in lagging strand

replication, this confirms that circle formation involves replication.

The results obtained with DnaG and with *terC* raise the possibility that DnaB may be involved in transposon circle formation. Both DnaG and DnaB appear necessary for optimal primer RNA synthesis on the lagging strand (Lu *et al*, 1996) and Tus shows specificity for DnaB. However, in similar experiments a *dnaBts* strain failed to show such delays in figure eight conversion (data not shown). This behaviour might reflect the existence of alternative pathways which operate in the absence of DnaB (e.g. use of an alternative helicase or, given the relatively short segment to be replicated, involvement of a helicase independent replication mechanism—see next paragraph). Indeed, it has recently been shown, for example, that non conventional branched molecules can be formed by transfer of a single IS*911* end (SET) between one end in an IR-IR junction and a second, isolated target IR (Loot *et al*, 2004). These partial transposition products carry a four-way DNA branched region similar to a Holliday junction but with a nick at the branch site. The structure can be resolved to generate forms which resemble the full transposition product. This involves branch migration and occurs by resolution of the second transposon strand in a process that requires the RecG helicase (Turlan *et al*, 2004). Although this reaction is probably much less efficient than the 'normal' complete transposition event and would not be expected to involve extensive DNA synthesis, it shows that several parallel and nonexclusive pathways may operate within the cell.

In the replicative model shown in Figure 4A, it is assumed that leading and lagging strand synthesis are coupled to give rise directly to a double stranded transposon circle. An alternative model would be that leading and lagging strand synthesis are not coupled and that transposon circles are first produced as single strand circles by simple extension from the 3′-OH of the donor backbone and 'extrusion' of the (unreplicated) single strand circle. The circles could then be processed to double strand circles in a second subsequent replication step similar to that which generates double strand RFII forms from circular single strand phage DNA following infection (Kornberg and Baker, 1992). Several levels of complexity for priming phage second strand synthesis are known. All involve initiation at a short hairpin structure in the phage genome. In the case of IS*911*, this could be provided by the 36bp abutted terminal IRs. In phage G4, for example, the DnaG primase alone accomplishes the priming step which necessitates a hairpin structure (Fiddes *et al*, 1978; Godson *et al*, 1978) close to the triplet 5′-CTG where RNA initiates (Hiasa *et al*, 1990). Such sequences can be found in IS*911* for both polarities of IR attack. Note that single strand circles have been observed for IS*91 in vivo* (Del Pilar Garcillan-Barcia *et al*, 2001) and are thought to be intermediates in the rolling circle transposition mechanism used by this IS. This type of alternative mechanism involving a single strand circular intermediate is at present under investigation.

Another question raised by these results is whether the presence of bound Tpase is required for mobilisation of host proteins and the formation of a replisome. For bacteriophage Mu, the presence of Tpase, MuA, in the synaptic complex is necessary to prepare the template for the initiation of DNA synthesis (Nakai and Kruklitis, 1995). The MuA complex is implicated in protecting the strand transfer product and the forked junctions until host factors are recruited which then can serve as an initiator of a 'cascade' of events leading to DNA replication. For IS*911*, the Tpase does not appear to be essential for initiating the replication step since purified deproteinised figure-eight intermediates were resolved following transformation into strains devoid of IS*911* proteins (Turlan *et al*, 2000). Nevertheless, we cannot exclude that Tpase bound at the junction created after the first strand transfer affects or facilitates the following steps in a quantitative manner by blocking the action of inappropriate enzymes as has been suggested for phage Mu (Nakai and Kruklitis, 1995).

While the results presented here clearly demonstrate that IS*911* circular transposition intermediates are produced from the figure-eight molecules by a replicative mechanism, many questions remain to be answered. Most importantly, the enzymology of the process has yet to be clarified. The DNA polymerase(s), clamp loading protein(s) and perhaps helicase(s) are yet to be identified and regulatory processes involved in the recombination-replication switch require analysis. Moreover, although circle formation involves replication, we have yet to determine whether this involves concomitant leading and lagging strand synthesis. In spite of the uncertainties inherent to the complexity of these events, the results presented here demonstrate that IS*911* and by extension, other members of the IS*3* family, have adopted a transposition mechanism different from other well known transposons such as IS*10*, IS*50*, Tn*7* and bacteriophage Mu. This strategy can be viewed as a 'copy-paste' mechanism compared to the 'cut and paste' mechanism involved in IS*10*, IS*50*, Tn*7* transposition or the replicative cointegrate formation pathway involved in Mu transposition and represents an alternative transposition paradigm. It should also be noted that this mechanism is likely to occur for many other IS families which also transpose using intermediates involving abutted IR-junctions.

Materials and methods

Bacterial strains and media

The strains for kinetic experiments were MC4100 (*araD*139Δ(*argF-lac*) u169 *rpsL*150 *relA*1 *rlbB*5301doC1 *ptsF*25 *rbsR*), or MC4100 *dnaG*ts. The strain used for *in vivo* ^{3}H-thymidine incorporation was LN2667 (*thi thy leu deoB supE SmR* (*srl*::Tn*10*) *recA*1). The *tus* strain used in Tus/*ter* experiment was LN4082 (HfrC del(*tus*:Ap) *thi thy leu deoB supE*). Cultures were grown in Luria broth supplemented with appropriate antibiotics. For thymidine incorporation experiments, cultures were grown in VB medium (for 1 l: 3.5 g $NaNH_4HPO_4$, 10 g K_2HPO_4, 2 g citric acid, 0.2 g $MgSO_4$) supplemented with thymine (50 μg/ml), thiamine (100 μg/ml), glucose (4 mg/ml), leucine (20 μg/ml), casamino acids (1 mg/ml), and appropriate antibiotics.

Plasmids

Plasmids supplying transposition functions in trans. Plasmids pAPT110 (which carries no IS*911* genes) and pAPT111 (which expresses OrfAB Tpase under the control of a p_{lacUV5} promoter) have been described previously (Polard and Chandler, 1995).

Transposase substrates. Kinetic experiments: Plasmid pLH39 used in these experiments, a p15A-based plasmid carrying an independent OrfAB gene under control of p_{lacuv5}, was constructed by cloning an IS*911*-derived transposon bearing a lacZ gene into pAPT111 (Polard and Chandler, 1995). For *in vivo* DNA-labelling experiments, plasmid pPR4, a derivative of a temperature-sensitive replication mutant of pSC101, was constructed as follows: the

*Eco*RI–*Bam*HI fragment from the temperature-sensitive pSC101-(Hashimoto-Gotoh and Sekiguchi, 1977) derived plasmid pGB2 (Churchward *et al*, 1984) was inserted between *Eco*RI and *Bam*HI sites of plasmid pAPT99 containing chloramphenicol gene between IRL and IRR (Polard and Chandler, 1995).

Experiments with terminator terC: The parental plasmid pDV44 was obtained by *Bal*I–*Bal*I fragment exchange of wild-type IRL of pPR4 described above, by a 5′-CA to 5′-TC terminal dinucleotide mutant of IRL (IRL*) from plasmid pAPT177 (Polard and Chandler, 1995).

terC sequences were obtained by annealing two 36 bp complementary oligonucleotides and introduced either outside (*Eco*RI site) or inside (*Xho*I site) an IS*911*-derived transposon in both orientations. The sequence of the *terC* sites used here was: 5′-AATATAGGATGTTGTAACTAATAT-3′. Additional restriction site sequences were added at both ends to facilitate cloning into the *Eco*RI or *Xho*I sites of pDV44.

Both orientations of *terC* (permissive or non permissive for leading strand replication) were obtained in the *Eco*RI site to generate pDV45 and 46 (Table I). Insertions into the *Xho*I site generated pDV42, 41, and 43.

To generate IRR* terminal dinucleotide mutations, a *Bal*I–*Bal*I fragment exchange of an IRR* from plasmid pAPT178 (Polard and Chandler, 1995) into pDV41, 42, and 43 was used to generate pDV48, 49, and 50, respectively. These plasmids are schematised in Table I. Note that the *Eco*RV restriction site within the donor backbone was removed during the construction of the IRR* variant plasmids. In these cases, the transposon and Tpase donor plasmids migrate as linear molecules at the same position in this gel (Figure 7C).

Alkaline lysates were prepared as described (Birnboim and Doly, 1979). Cleared lysates were prepared as described (Clewell and Helinski, 1969), with the following modifications: the cleared supernatants collected were concentrated by isopropanol precipitation and resuspended in 100 µl of TE buffer and purified by using a Qiaquick kit as described by the supplier (Qiagen).

Kinetics of figure-eight and circle formation in vivo

Plasmid pLH39 was used in these experiments. The culture was grown at 42°C to inactivate any residual Tpase activity (OrfAB is temperature sensitive). At $t=0$ ($OD_{600}=0.6$), the population was transferred into LB medium pre-warmed at 30°C and production of Tpase was induced with IPTG. Samples were withdrawn at different times, growth was stopped by transfer in a sodium azide mixture (50 mM, final concentration) in ice, and cleared lysates were then made. The samples were cleaved with *Bam*HI and separated by agarose gel electrophoresis (1% agarose, TAE buffer); the gel was dried at 60°C and used directly for hybridisation. A ^{32}P 5′-labelled oligonucleotide complementary to the 5′ end of the *orfAB* gene also present within the IS*911*-derived transposon was hybridised for 12–15 h and analysed using a Fuji BAS1000 phosphorimaging system.

Kinetics of figure-eight to circle conversion in vivo

Cells carrying pLH39 were grown at 30°C in the presence of IPTG to induce Tpase synthesis and figure-eight formation. At $t=0$ ($OD_{600}=0.6$), the culture was centrifuged and resuspended in fresh LB medium pre-warmed at 42°C to inactivate Tpase. Samples were withdrawn at different times, growth was stopped by transfer in a sodium azide mixture (50 mM) in ice, and cleared lysates were then made. The samples were cleaved with *Bam*HI and separated by agarose gel electrophoresis (1% agarose, TAE buffer). The values were calculated as follows: the intensities of figure-eight and circle bands were measured with a Fuji BAS1000 phosphorimaging system coupled to the PCBas software and the sum was used to define a value of 100%. The level of parental plasmid was used to normalise sampling variations from well to well. Since the parental plasmid continues to replicate during the experiment whereas transposition intermediates are no longer produced, parental plasmid DNA increases over time with the growth rate of the culture. This increase was also taken into account in the normalisation.

For experiments using mutants defective for the DnaG primase, after production of figure-eight molecules at 30°C (Tpase OrfAB and DnaG active), the conversion of figure-eights to circles of a wild-type strain was compared with the *dnaG*ts strain at 42°C. The samples were separated by agarose gel electrophoresis (1% agarose, TAE buffer) and treated as above.

^{3}H-thymidine DNA labelling in vivo

A culture of a *thyA*$^{-}$ strain carrying the temperature-sensitive plasmid pPR4 as a transposon vector was grown at 30°C in 100 ml of minimal VB medium complemented with thymine, thiamine, and casamino acids. OrfAB synthesis was induced with 1 mM IPTG and the culture was grown to an OD_{600} of 0.6. The culture was concentrated into 10 ml of the same fresh medium but depleted in thymine and IPTG, pre-warmed at 45°C in order to inactivate OrfAB production and replication of the transposon-carrying plasmid. After 2 min of incubation to deplete the internal pool of cold thymine, 500 µl of [methyl, 1′, 2′-^{3}H]thymidine (113 Ci/mmol) was added for an additional 10 min. Alkaline lysates were then prepared (Qiagen miniprep kit), plasmid DNA was then digested with *Eco*RV and separated by agarose gel electrophoresis. After migration, the gel was equilibrated in 20 volumes of 95% EtOH for 30 min. This step was repeated twice to eliminate a maximum amount of water present in the gel. The gel was then soaked in a solution of autoradiography enhancer (NEN) for 3 h, and rinsed in water for 1 h. The gel was dried at 60°C under vacuum and exposed to a Kodak Biomax film for about a week.

Circle formation in the presence of terC sites in tus^{+} or tus^{-} strains

IR donor mutants were used to obtain a unique figure-eight population to orient the replication fork movement in a unique polarity during the resolution. IRL* or IRR* donor mutants were tested separately with different positions and orientations of *terC* sites inside (pDV41, 42, 43 with IRL*, or pDV48, 49, 50 with IRR*) or outside IS*911* (pDV45 and 46). pDV43 and 50 harbour two t*erC* sites in tandem. The appearance of circles was analysed for all plasmids in both tus^{+} or tus^{-} strains. Plasmid DNA was digested with *Eco*RV which cleaves once within the transposon, once within the donor backbone, and once in the transposon donor plasmid, except for IRR* variants which lack the *Eco*RV site near IRR within the donor plasmid backbone. In this case, the transposon and Tpase donor plasmids migrate as linear molecules at the same position in gel (Figure 7C).

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