

RecFOR proteins are essential for Pol V-mediated translesion synthesis and mutagenesis

Shingo Fujii¹, Asako Isogawa¹
and Robert P Fuchs*

Genome Instability and Carcinogenesis, CNRS FRE2931, Marseille, France

When the replication fork moves through the template DNA containing lesions, daughter-strand gaps are formed opposite lesion sites. These gaps are subsequently filled-in either by translesion synthesis (TLS) or by homologous recombination. RecA filaments formed within these gaps are key intermediates for both of the gap-filling pathways. For instance, Pol V, the major lesion bypass polymerase in *Escherichia coli*, requires a functional interaction with the tip of the RecA filament. Here, we show that all three recombination mediator proteins RecFOR are needed to build a functionally competent RecA filament that supports efficient Pol V-mediated TLS in the presence of ssDNA-binding protein (SSB). A positive contribution of RecF protein to Pol V lesion bypass is demonstrated. When Pol III and Pol V are both present, Pol III imparts a negative effect on Pol V-mediated lesion bypass that is counteracted by the combined action of RecFOR and SSB. Mutations in *recF*, *recO* or *recR* gene abolish induced mutagenesis in *E. coli*.

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Introduction

DNA damage is continuously generated inside cells. In addition to efficient and accurate DNA repair mechanisms that act before replication to remove most lesions, cells have evolved mechanisms that allow them to tolerate lesions at the replication fork. Such tolerance mechanisms comprise two main strategies: translesion synthesis (TLS), a potentially mutagenic strategy during which specialized DNA polymerases copy the damaged template, and damage avoidance strategies that are related to homologous recombination and are therefore essentially accurate. Experimental data on the structure and dynamics of a replication fork encountering blocking lesions are still limited. The current view is that when lesions

are present in the parental DNA, the replication fork keeps going leaving gaps opposite the lesion sites in both lagging and leading strands (for a recent review, see Langston and O'Donnell, 2006; Lehmann and Fuchs, 2006). It turns out that this model, which has been proposed almost four decades ago by Rupp and Howard-Flanders, (1968) has recently received renewed attention on the basis of several *in vivo* (Pages and Fuchs, 2003; Lopes *et al*, 2006) and *in vitro* (Heller and Marians, 2006) reports. The gaps left in the newly synthesized strands behind the moving fork are subsequently filled-in either by TLS or by a recombination-type mechanism (*recF* pathway). To function in TLS, Pol V requires a physical contact with the tip of a RecA filament formed on single-stranded DNA (Dutreix *et al*, 1989; Fujii *et al*, 2004; Schlacher *et al*, 2006). It is thus interesting to note that both gap-filling mechanisms require a common intermediate, namely a RecA-covered ssDNA filament. Despite the fact that cells contain similar amounts of ssDNA-binding protein (SSB) and RecA proteins (Karu and Belk, 1982; Williams *et al*, 1984), SSB binds preferentially to ssDNA as a consequence of its much higher affinity for ssDNA than RecA (Arenson *et al*, 1999; Ehn *et al*, 2001). In all species including phages, formation of a recombinase-ssDNA filament requires the function of the so-called recombination mediator protein(s) that loads the cognate recombinase protein on single-stranded DNA covered by the cognate SSB (Gasior *et al*, 2001). In *Escherichia coli*, the *recFOR* gene products are thought to function as mediator proteins based on the following observations: (1) strains that carry the mutant *recF*, *recO* or *recR* allele show a delay in SOS induction kinetics (Hegde *et al*, 1995; Whitby and Lloyd, 1995) and UV sensitivity, possibly reflecting the observed degradation of nascent DNA strands, that are normally prevented by RecA function (Courcelle and Hanawalt, 2003; Chow and Courcelle, 2004) (2) specific *recA* alleles (e.g., *recA803*) are able to suppress the *recF*, *recO* or *recR* phenotype (Wang *et al*, 1993). *In vitro*, RecA803 protein can dissociate SSB from ssDNA efficiently compared with wt RecA (Lavery and Kowalczykowski, 1992). (3) RecOR proteins are capable to mediate *in vitro* an exchange reaction from SSB to RecA on ssDNA (Umezū *et al*, 1993). A role of RecF protein in this process has only been emerging recently (see below). Decades of work on *recF*, *recO* and *recR* genes have shown that RecFOR proteins are intimately involved in the establishment of a RecA filament when SSB limits RecA nucleation (Madiraju *et al*, 1988; Sawitzke and Stahl, 1992; Clark and Sandler, 1994; Whitby and Lloyd, 1995; Kuzminov, 1999). RecA filament formation can be viewed as a dynamic process that entails two phases: a rate-limiting nucleation step, followed by a rapid extension step; although continuous assembly and disassembly of monomers occurs at both ends in the presence of ATP, the net growth of the filament proceeds in the 5'–3' direction by virtue of a higher association rate at the 3' extending end (Register and Griffith, 1985; Shan *et al*, 1997; Bork *et al*, 2001a; Joo *et al*, 2006). Both SSB and RecX proteins modulate the formation of the RecA

*Corresponding author. Genome Instability and Carcinogenesis, CRNS, FRE 2931, 31, chemin Joseph Aiguier, 13402 Marseille cedex 20, 13402, France. Tel.: +33 4 9116 4271; Fax: +33 4 9116 4168; E-mail: fuchs@ibsm.cnrs-mrs.fr

¹These authors contributed equally to this work

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filament. Whereas SSB proteins at sub-stoichiometric concentrations favor the RecA filament extension phase by dissolving secondary structures, stoichiometric concentrations of SSB block the RecA nucleation phase. RecOR proteins allow to overcome the SSB-mediated nucleation block. On the other hand, RecF protein specifically overcomes the effect of RecX that otherwise blocks the RecA filament extension phase (Lusetti *et al*, 2006).

We have previously determined the optimal conditions for Pol V-mediated bypass using single-stranded circular templates with a single lesion. The use of a circular template was found to be instrumental by supporting the stable loading of the beta-clamp and the formation of a dynamic RecA filament in the presence of ATP (Fujii *et al*, 2004). Under these conditions, Pol V extends the primer several nucleotides past the lesion generating a 'TLS patch'. Synthesis of such a TLS patch by the specialized DNA polymerase in a single binding event conceals the distortion created by the lesion, thus preventing degradation of the nascent primer by the proofreading activity associated with the replicative polymerase (Fujii and Fuchs, 2004; Fujii *et al*, 2004). However, physiologically relevant concentrations of SSB totally inhibit the Pol V-mediated TLS reaction (Fujii *et al*, 2004). In the present paper, we show that in the presence of saturating concentrations of SSB, efficient Pol V-mediated lesion bypass is achieved in the presence of RecFOR proteins. In addition, when Pol III and Pol V are simultaneously present in the reaction mix, Pol III appears to severely hinder Pol V-mediated TLS. Addition of RecFOR and SSB proteins appears to reverse the adverse effect of Pol III on Pol V-mediated bypass. In good agreement with these *in vitro* data, inactivation of either *recF*, *recO* or *recR* gene inhibits Pol V-mediated UV-induced mutagenesis in a forward mutation assay to the same extent as a *umuDC* mutant.

Results

Forward mutation assay and TLS in *recF*, *recO* and *recR* mutant strains

In *E. coli*, induced mutagenesis is known to depend upon several key proteins, namely Pol V, a Y-family polymerase responsible for the bypass of most lesions, the beta-clamp, the general replication processivity factor, and RecA protein (for a review, see Fuchs *et al*, 2004). Using the Weigle mutagenesis protocol, it was reported that a host *recF* mutation severely inhibits UV and apurinic site mutagenesis (Schaaper *et al*, 1982; Wood and Stein, 1986). In contrast, conflicting results have been reported using different chromosomal reversion assays. Indeed, while reversion assays based on *hisG4* and *argE3* alleles showed that *recF* and *recR* mutants are normal with respect to UV-induced mutagenesis (Kato *et al*, 1977; Mahdi and Lloyd, 1989; Clark and Sandler, 1994), the reversion frequency of the *trpE65* allele was found to be severely reduced in all three *recF*, *recO* and *recR* mutant strains (Liu *et al*, 1998). Here, we show that inactivation of either *recF*, *recO* or *recR* gene strongly reduces UV-induced

mutagenesis using a chromosomal mutation assay as well as a plasmid-based assay capable of directly measuring Pol V-mediated translesion synthesis opposite a single UV-induced lesion, namely the TT(6-4) photoproduct *in vivo*.

UV-induced mutagenesis: *rif^R* assay. Induction of rifampicin-resistant colonies following UV irradiation was monitored as described in Materials and methods. The frequency of induced mutations was determined at various UV doses in wild-type strain and in isogenic *recF*, *recO* and *recR* mutant allele strains. The wild-type strain exhibits a robust induction of *rif^R* mutants, reaching about 3×10^{-6} at a UV dose corresponding to 1% survival. This value corresponds to ≈ 100 -fold increase above background ($\approx 3-5 \times 10^{-8}$) (Figure 1A). For all three *rec⁻* strains, the induction of UV-induced mutants was 5- to 10-fold reduced when compared with the

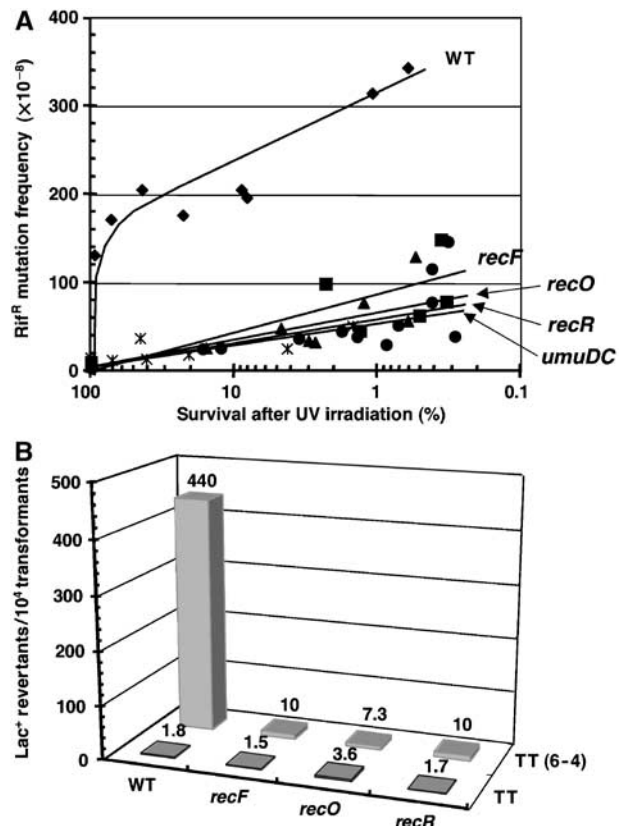


Figure 1 UV-induced mutagenesis. (A) Rifampicin resistance chromosomal mutagenesis assay. The frequency of UV-induced *Rif^R* colonies is plotted against survival in wild-type, *recF*, *recO*, *recR* and *umuDC* strains in the MG1655 background (see Materials and methods for additional details). Plotting mutagenesis against UV survival rather than UV dose allows one to take into account the UV sensitivity of *recF*, *recO* and *recR* strains. (B) Mutagenesis induced by a single TT(6-4) lesion. A single TT(6-4) photoproduct was introduced in a double-stranded plasmid vector, as reported previously (Becherel and Fuchs, 1999). The adduct is located in the *lacZ* (α) gene in a way that allows direct monitoring of mutagenic bypass by a change in colony color on *lacZ* indicator plate (see Materials and methods). All strains contain a low-copy number plasmid pRW134 expressing Pol V (Frank *et al*, 1996) at a level similar to that found in UV-induced cells. The numbers represent the average mutation frequency determined from three independent experiments.

wild-type strain, reaching a level similar to that observed in a *umuC* mutant (Figure 1A).

Single TT(6-4)-induced mutation frequency. We also implemented a specific mutation assay involving a single TT(6-4) lesion located on a double-stranded plasmid vector. Both error-free and mutagenic bypass of this lesion were shown to depend entirely on Pol V (Becherel and Fuchs, 1999). The expression of Pol V calls for a functional SOS system at both the transcriptional and post-translational levels. In order to circumvent the difficulty related to the fact that *recF*, *recO* or *recR* strains are known to have delayed SOS induction kinetics (Hegde *et al*, 1995; Whitby and Lloyd, 1995) compared to a wild-type strain, we transformed our strains with a low-copy vector that constitutively expressed Pol V at a level similar to SOS-induced cells (Frank *et al*, 1996). We then introduced the TT(6-4) lesion-containing plasmid in these strains to score mutagenic lesion bypass directly on indicator plates as blue colonies (see Material and methods). The mutant colony fraction reached 4.4% in the wild-type strain, whereas this response was totally abolished in the *recF*, *recO* or *recR* strains (Figure 1B), demonstrating the direct involvement of RecFOR in Pol V-mediated TLS. In the wild-type strain, the mutagenic response is completely dependant on the presence of the Pol V-encoding plasmid (data not shown).

Reconstitution of the TLS reaction in the presence of Pol III HE, Pol V, SSB and RecA

Physiological concentrations of SSB completely block Pol V-mediated TLS: this block is relieved by the addition of RecFOR proteins. *In vivo*, ssDNA gaps are formed when the replicative DNA polymerase dissociates from its template, generating, in most cases, a 3'-end located at the position immediately preceding the lesion site (position L-1). To mimic the formation of such a primer by Pol III *in vitro*, we used a primer that ends 8 nucleotides upstream of the lesion site, allowing it to be elongated by Pol III (running start experiments). We implemented the previously optimized TLS assay conditions involving a single-stranded circular template primed with a ³²P-labeled oligonucleotide in the vicinity of a single DNA lesion (G-AAF adduct) (Fujii *et al*, 2004). This substrate was incubated successively with purified SSB (5 min) and RecA (3 min) proteins in quantities that are sufficient for full coverage of the single-stranded DNA present in the assay. Following addition of RecFOR proteins (5 min), the substrate was incubated in the presence of Pol III HE, followed by Pol V (quantities and incubation time as indicated in Figure 2 and in Materials and methods) before the reaction is stopped for PAGE analysis.

The concentrations of RecF (0.1 μM), RecO (0.2 μM) and RecR (10 μM) that achieve optimal Pol V bypass have been determined by various titration experiments (see Supplementary Figures S1, S2, S4 and S5). As seen in Figure 2A, in the presence of the RecFOR proteins, a robust time-dependent appearance of full-length TLS product (TLS0) can be observed reaching ≈60% of bypass at 1 h. A strong pause site corresponding to the position preceding the lesion site (L-1) is rapidly observed and becomes less intense over time when bypass occurs. In contrast, in the absence of RecFOR proteins, the primer is rapidly elongated until position L-1, whereas essentially no TLS takes place under these

conditions. Indeed, the faint full-length band at position TLS0 is constant over time and corresponds to a small contamination with non-modified substrate (≈0.2%). In contrast, the faint band at position TLS-1 slightly increases over time, and is due to a weak frameshift bypass activity mediated by Pol III HE (≈0.8% at 80 min).

In conclusion, in the presence of Pol III HE and saturating concentration of SSB and RecA, RecFOR proteins are necessary to reach high levels of Pol V-mediated lesion bypass. We suggest that RecFOR proteins are required for RecA filament formation in the presence of physiologically relevant concentrations of SSB. While under normal conditions, the cellular concentrations of RecA and SSB monomers are comparable, when SOS is induced, RecA concentration increases about 10-fold. However, given the much higher affinity for binding to ssDNA of SSB (Ehn *et al*, 2001) compared with RecA (Arenson *et al*, 1999), the competition is still largely in favor of SSB.

Pol V-mediated TLS in the presence of SSB: RecOR proteins are absolutely necessary, whereas RecF further stimulates the reaction. None of the RecF, RecO or RecR protein taken alone or in combination of two is able to stimulate TLS above background level, except for the RecOR combination (Figure 2B). Indeed, the RecOR combination allows the TLS reaction to reach a level very significantly above background (3.7% for a 10 min incubation time with Pol V). Previously published biochemical data have established that the RecOR complex functions to speed up the formation of a RecA filament on ssDNA prebound by the SSB protein (Umezumi and Kolodner, 1994; Shan *et al*, 1997; Bork *et al*, 2001b). In these experiments, addition of RecF had either no or even a negative effect on the capacity of RecOR to load RecA. In contrast, we found that addition of RecF (0.1 μM) further stimulated the TLS reaction about five-fold (Figure 2B). Similarly, by monitoring the ATPase activity of the RecA filament, it was recently shown that RecF stimulates RecA filament formation on ssDNA gaps coated with SSB protein (Morimatsu and Kowalczykowski, 2003).

TLS in the presence of Pol III and Pol V polymerases: effect of SSB. We wanted to investigate the effect of SSB concentration on the efficiency of the TLS reaction in the presence and absence of RecFOR proteins. In the absence of RecFOR proteins, low concentrations of SSB (10 nM, i.e., 5–10% of the stoichiometric amount) stimulated Pol V activity by promoting RecA filament extension, whereas increasing amounts of SSB (i.e., up to stoichiometric amounts) totally inhibited the bypass reaction, as shown previously (Fujii *et al*, 2004). This observation reflects the two aspects of SSB action: although SSB strongly inhibits the nucleation phase of RecA filament assembly, it stimulates the extension phase by dissolving the secondary structures in ssDNA that would otherwise impede filament growth (Kowalczykowski and Krupp, 1987; Kowalczykowski *et al*, 1987; Lavery and Kowalczykowski, 1992). Surprisingly, in the presence of RecFOR proteins, increasing amounts of SSB very efficiently stimulated the bypass reaction, reaching levels that are in fact higher than in the absence of SSB and RecFOR proteins (Figure 2C). Kinetics of lesion bypass experiments were performed to further investigate the nature of this effect (see below and Figure 4).

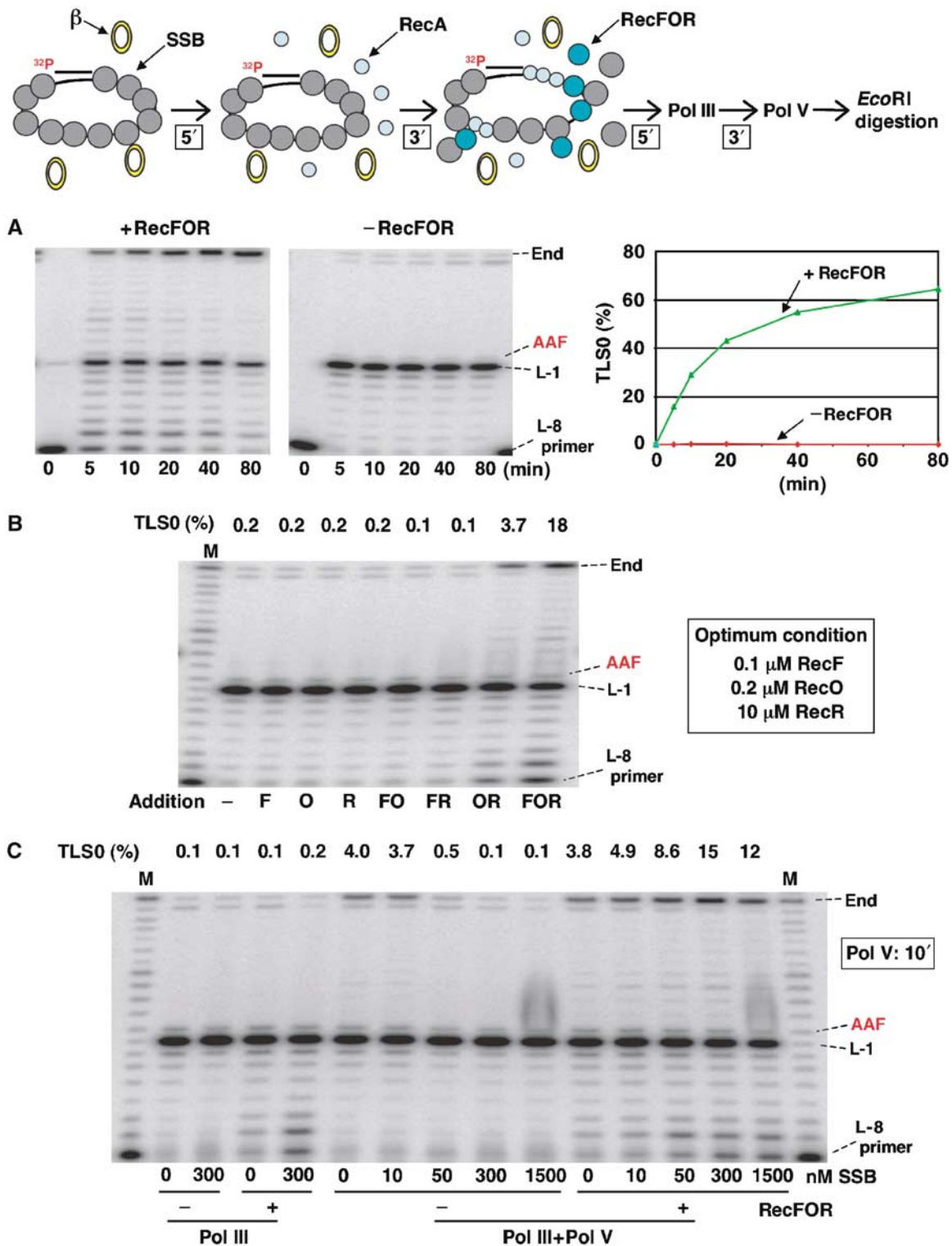


Figure 2 Pol III/Pol V-mediated TLS reaction in the presence of RecA and SSB proteins. (A) RecFOR proteins efficiently allow Pol III/Pol V-mediated TLS. The concentrations of RecA and SSB tetramers to fully saturate the ssDNA template present in the assay (2 nM of single-stranded circular plasmid 2.7-kb long) are 1.8 μ M and 160 nM, respectively. Assays are performed at 2 μ M and 300 nM of RecA and SSB tetramers, respectively. In the presence of optimal concentrations of RecFOR proteins, a robust TLS reaction is observed (> 60%) at 80 min incubation. In contrast, in the absence of RecFOR proteins, essentially no bypass is observed, as the TLS0 band observed in the gel corresponds to a 0.2% contamination with lesion-free template. Percentage of TLS0 is calculated as specified in Materials and methods. (B) None of the RecF, RecO or RecR proteins alone or in combination of two have an effect on TLS, except for the RecOR combination. Further addition of RecF increases the reaction efficiency. Incubation time: Pol III, 3 min; Pol V, 10 min. (C) When both Pol III and Pol V are present, high concentrations of SSB and optimal concentrations of RecFOR proteins are necessary to yield optimal bypass conditions. In the absence of RecFOR proteins, no or low amounts of SSB protein (10 nM, i.e., 5–10% of the stoichiometric amount) yield optimal bypass. However, a four-fold higher bypass reaction is observed in the presence of RecFOR and saturating amounts of SSB tetramer. Incubation time: Pol III, 3 min; Pol V, 10 min; in the absence of Pol V, incubation time with Pol III is 13 min.

Role of the 5'-primer end (matched versus tailed) in RecFOR stimulation of Pol V-mediated TLS

Using the ATPase activity as a reporter for RecA filament formation, the presence of a perfectly matched 5'-ds/ss junction was reported to be important for RecF-mediated stimulation of RecA filament formation on SSB-coated ssDNA gaps (Morimatsu and Kowalczykowski, 2003). Indeed, the presence of a 5'-unpaired region (5'-tail) was shown to abolish RecF stimulation (Morimatsu and Kowalczykowski, 2003). We wanted to investigate the effect of a 5'-tail on Pol V-mediated TLS. Compared to a perfectly matched 5'-ds/ss junction, we observed a three-fold reduction with the 5'-tailed structure (compare Figures 2B and 3A). However, we cannot attribute this reduction specifically to RecF, as a similar reduction in the stimulation was observed with RecOR alone (Figure 3B). In fact, with the tailed substrate, when compared to RecOR alone, addition of RecF stimulated very significantly the kinetics of TLS (Figure 3B).

Pol V-mediated bypass reaction is severely inhibited by the presence of Pol III HE: RecFOR and SSB proteins can reverse the adverse effect of Pol III

Using concentrations of RecA (2 μ M) and SSB (10 nM) previously determined for optimal Pol V-mediated bypass, we observed that the presence of Pol III HE in the reaction mixture severely decreases (\approx 10-fold decrease) the kinetics and extent of the TLS reaction (Figure 4). Addition of RecFOR proteins allowed the TLS reaction to recover to levels similar to those observed in the absence of Pol III HE (Figure 4). However, a distinct effect was observed in terms of kinetics depending on SSB concentration. At low SSB concentrations, we observed full recovery of TLS but with a reduced kinetics, whereas at high SSB concentrations, the kinetics of the TLS reaction was similar to that observed in the absence of Pol III (Figure 4). How can these effects be explained? Compared with the situation with Pol V alone, when Pol III is present, it may interfere with the Pol V-mediated bypass reaction by competing for primer template binding at position L-1 (Figure 6A). As binding of Pol III is non-productive for lesion bypass, Pol III may be expected to interfere with Pol V binding. However, the large decrease in TLS efficiency is not compatible with a simple model of competition for substrate binding. Indeed, at constant Pol V concentration (100 nM), a large increase in Pol III concentration, from 4 to 60 nM, only further reduced TLS efficiency by a factor of two (data not shown). We hypothesized that binding of Pol III actually disturbs the 3'-tip of the RecA filament that is essential for Pol V bypass. Indeed, it is unlikely that binding of Pol III to sites within the RecA filament that are distant from its lesion proximal 3'-tip would influence Pol V-mediated TLS activity. Therefore, RecFOR proteins together with SSB appear to stimulate the TLS reaction by allowing proper and rapid reconstruction of the tip of the RecA filament that is perturbed by (repeated) binding of Pol III HE (Figure 6A). The precise mechanism of RecA filament reconstruction is presently unknown.

Bypass of the major UV-induced lesions: TT cyclobutane dimer (CPD) and TT(6-4) photoproduct

Experiments similar to those conducted with the single G-AAF adduct have been performed with substrates containing either a single TT CPD or TT(6-4) lesion (Figure 5). In the

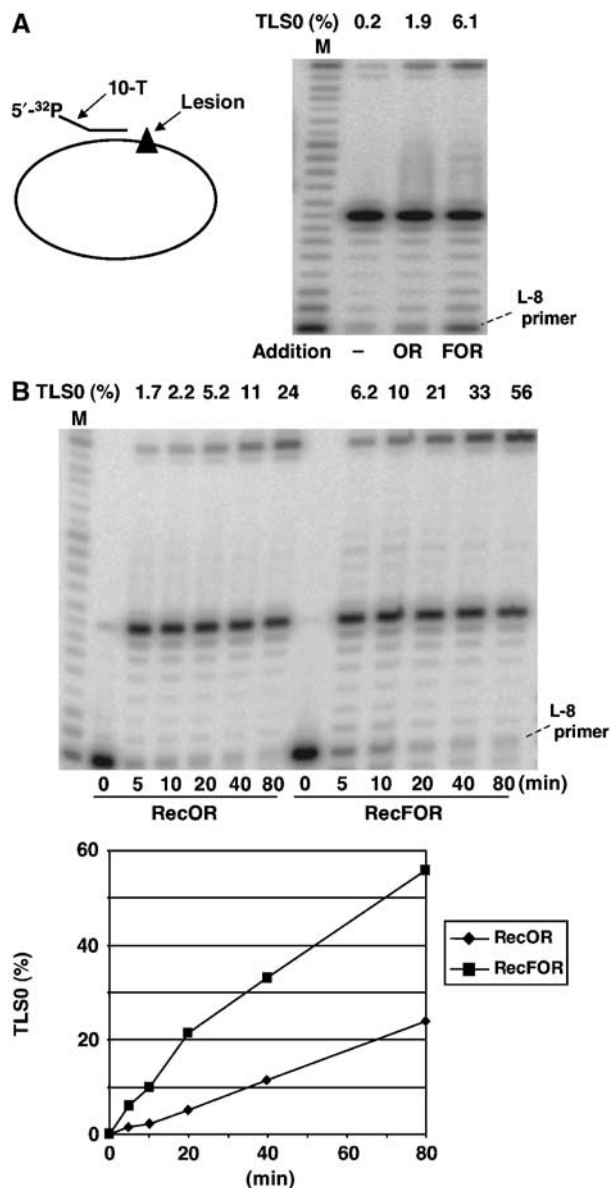


Figure 3 Effect of 5'-tailed primer on RecFOR stimulation of Pol V-mediated bypass. A primer containing 10 dT residues at its 5'-end was used. Incubation time: Pol III, 3 min; Pol V, 10 min. (A) A three-fold reduction in lesion bypass was observed when compared to the fully matched primer (see Figure 2B). (B) Despite an overall reduction in lesion bypass with the tailed versus the matched primer, RecF clearly stimulated the bypass reaction observed in the presence of RecOR, as seen with the perfectly paired primer (see Figure 2B).

absence of RecFOR proteins, no bypass is seen, as the weak band at the top of the gel represents a low fraction (\approx 2%) of lesion-free template contained in our construction. After correction for this contamination, a comparable time-dependent accumulation of full-length TLS product is observed for both UV-induced lesions. Under optimal conditions, bypass of all three lesions is comparable with the following slight differences in efficiencies: G-AAF > TT(6-4) > TT CPD.

Discussion and conclusion

In vivo, SSB and RecA are abundant proteins that exhibit affinity for single-stranded DNA. Given the much higher

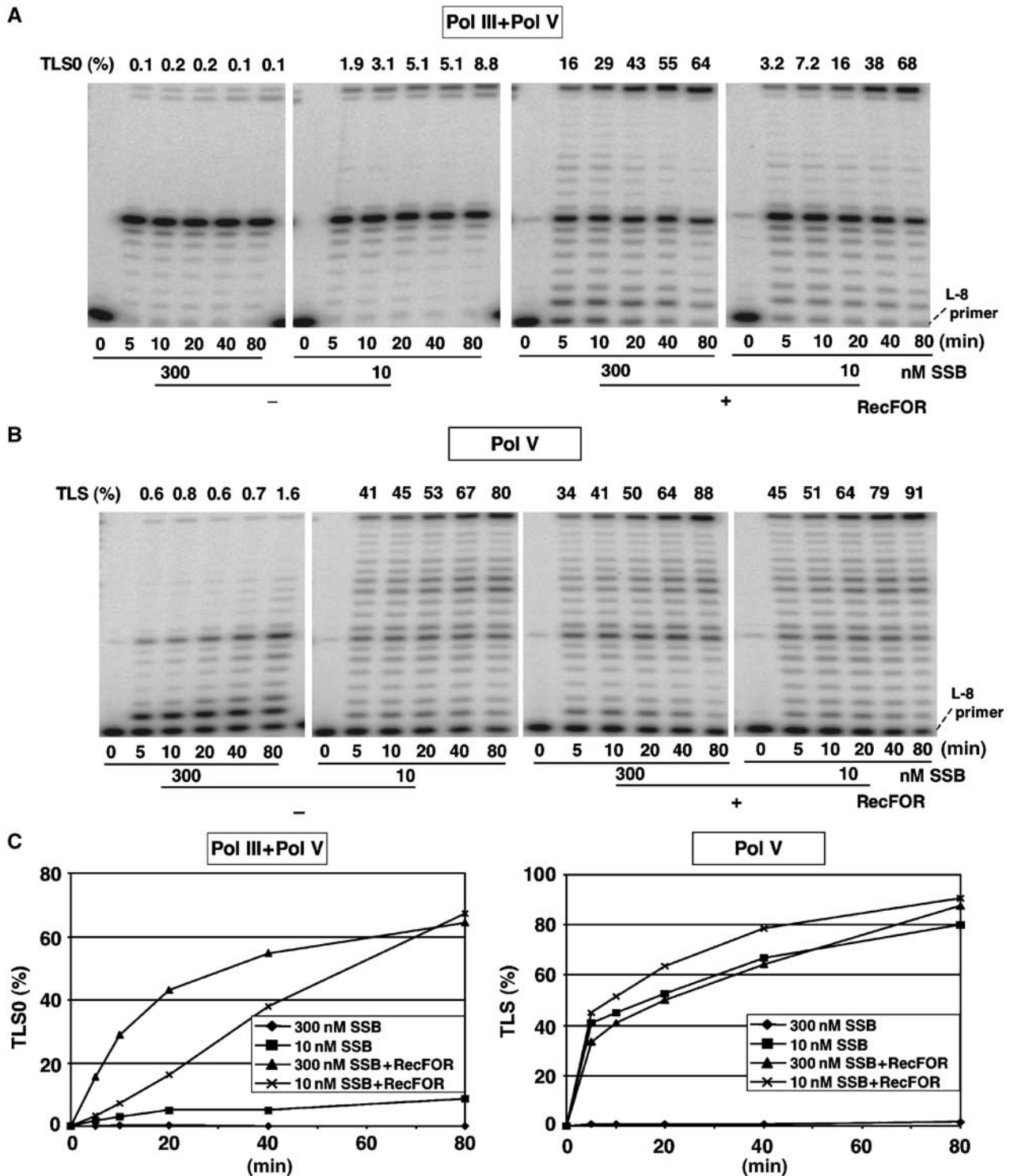


Figure 4 Kinetics of TLS as a function of SSB and RecFOR concentrations. (A) Pol III/Pol V-mediated TLS: in the presence of RecFOR, the bypass reaction kinetics is faster with saturating amounts of SSB compared with low amounts of SSB, whereas similar levels are reached at 80 min. In the absence of RecFOR, saturating amounts of SSB completely inhibit the reaction, whereas low amounts of SSB yield an ≈ 10 -fold reduced level of bypass compared with the + RecFOR conditions. (B) Pol V alone (no Pol III): in order to load the beta-clamp, we added purified gamma-complex clamp loader at 10 nM final concentration. In the presence of RecFOR, the bypass reaction is efficient with either low or high amounts of SSB. In the absence of RecFOR, the bypass reaction is efficient at low SSB concentrations, but is completely inhibited at high SSB concentrations. Under these conditions, the bypass reaction merely reflects the efficiency of RecA filament formation. (C) TLS0 kinetic curves under the conditions shown in (A, B).

affinity for ssDNA of SSB compared with RecA (Arenson *et al*, 1999; Ehn *et al*, 2001), ssDNA is preferentially covered by SSB, preventing RecA filament formation and thus inhibiting RecA filament-mediated cellular functions. A scenario similar

to the one seen in *E. coli* seen in most organisms: a given recombinase (RecA) requires the assistance of recombination mediator proteins (RecFOR) to dislodge the cognate SSB from ssDNA. Here, we demonstrate that RecFOR proteins are

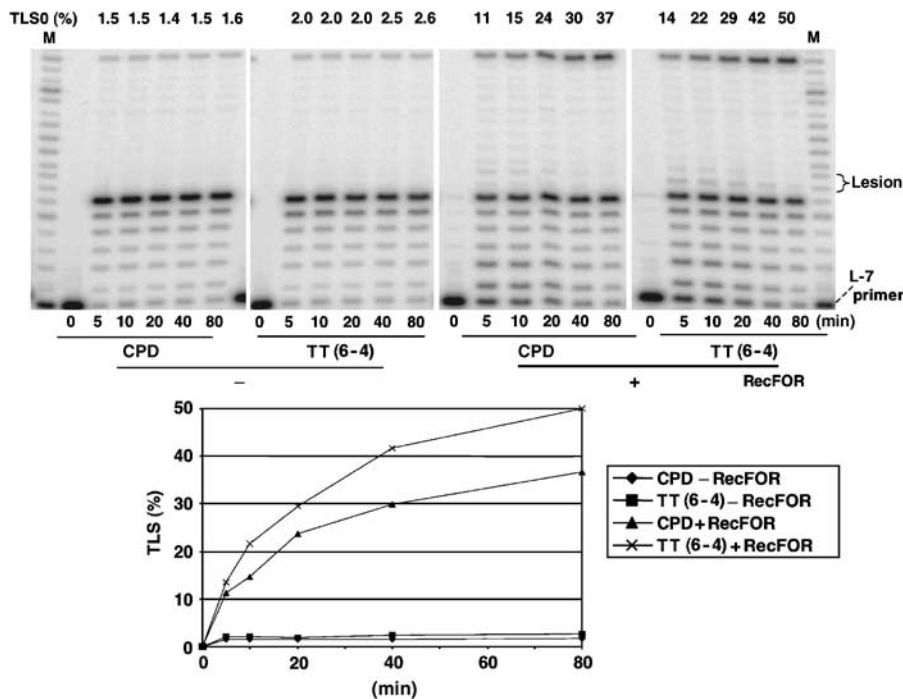


Figure 5 Lesion bypass of UV-induced TT lesions. The reaction conditions are the same as described for the G-AAF bypass in Figure 4A, except for the concentration of Pol III (96 nM instead of 62.7 nM) with saturating amounts of SSB (300 nM). Robust bypass is observed for both TT CPD and TT(6-4) lesions when RecFOR proteins are present. No bypass is observed in the absence of RecFOR; the weak TLSO band is due to a low ($\approx 2\%$) contamination by lesion-free template.

essential for efficient Pol V-mediated lesion bypass under conditions that mimic the *in vivo* situation, that is, in the presence of RecA and SSB proteins at concentrations that fully saturate the ssDNA substrate. None of the RecF, RecO or RecR protein alone or in any combination of two is able to activate Pol V-mediated TLS, except for the RecO plus RecR combination (Figure 2B). Addition of RecF to the RecOR combination further stimulates the TLS reaction, reaching an optimum at 0.1 μ M of RecF (Supplementary Figure S4). We suggest that RecFOR proteins trigger lesion bypass by allowing efficient formation of a RecA filament that is an essential cofactor for Pol V's TLS activity. The precise role of RecA in Pol V activation is still a matter of debate (see Pham *et al*, 2001; Reuven *et al*, 2001; Fuchs *et al*, 2004; Fujii *et al*, 2004; Schlacher *et al*, 2005). Recently, using short oligonucleotide templates, Goodman and co-workers suggested that efficient TLS requires activation of Pol V by a RecA filament in *trans* (Schlacher *et al*, 2006). However, in our hands, using a long single-stranded circular template, efficient Pol V lesion bypass activity does not rely upon the addition of a RecA filament in *trans* (Fujii *et al*, 2004).

The effect of RecFOR proteins on RecA filament formation in the presence of saturating amounts of SSB can be monitored by several assays: (1) an ATPase assay specific for the ssDNA.RecA complex; (2) a LexA or UmuD cleavage assay; (3) a homologous recombination assay based on joint molecule formation; and (4) the present assay monitoring Pol V-mediated TLS. While Pol V activation for TLS is thought to be specifically mediated by the 3'-tip of the filament (Fujii *et al*, 2004; Schlacher *et al*, 2006), all the other assays monitor the 'internal portions' of the RecA filament instead. For all RecA filament formation assays in the presence of SSB, the RecOR complex is found to be either essential or to strongly speed up

the reaction, whereas RecF acts as a modulator, stimulating or inhibiting the reactions depending on its concentration (see Supplementary Figure S2; Umezu *et al*, 1993; Shan *et al*, 1997; Xu and Marians, 2003). The role of RecF as a modulator rather than a core component of the recombination mediator apparatus is supported *in vivo* by the fact that *recF* phenotypes can partially be suppressed by RecR overexpression (Sandler and Clark, 1994) or by disruption of the *recX* gene (Lusetti *et al*, 2006).

How can we envision the role of RecFOR in RecA filament formation? RecA filament formation entails two basic steps: a rate-limiting nucleation step, followed by a rapid extension step. The RecOR complex by interacting with SSB.ssDNA (Griffin and Kolodner, 1990; Luisi-DeLuca and Kolodner, 1994) may displace the SSB filament, thus providing space for the RecA nucleation step. We envision that RecF either enhances RecOR function or assists the RecA nucleation step. In most *in vitro* assays published so far, RecF was reported to have either no or an inhibitory effect, except for two recent reports: (1) RecF specifically allows RecFOR to bind the ss/ds DNA junction at a fully base-paired 5'-terminus (Morimatsu and Kowalczykowski, 2003) and (2) RecF protein physically binds to RecX protein and antagonizes the effect of RecX that otherwise inhibits RecA filament formation (Lusetti *et al*, 2006). We show an additional property for RecF, namely its capacity to stimulate PolV-mediated bypass (Figures 2 and 3) independently of the presence of a fully paired ss/ds 5'-junction suggesting that the nucleation step does not require such a junction but can take place at any place along the ssDNA stretch instead. Alternatively, a direct role of RecF in Pol V-mediated TLS cannot be excluded.

On the basis of the biochemical data presented here and previously (Fujii and Fuchs, 2004; Fujii *et al*, 2004), we

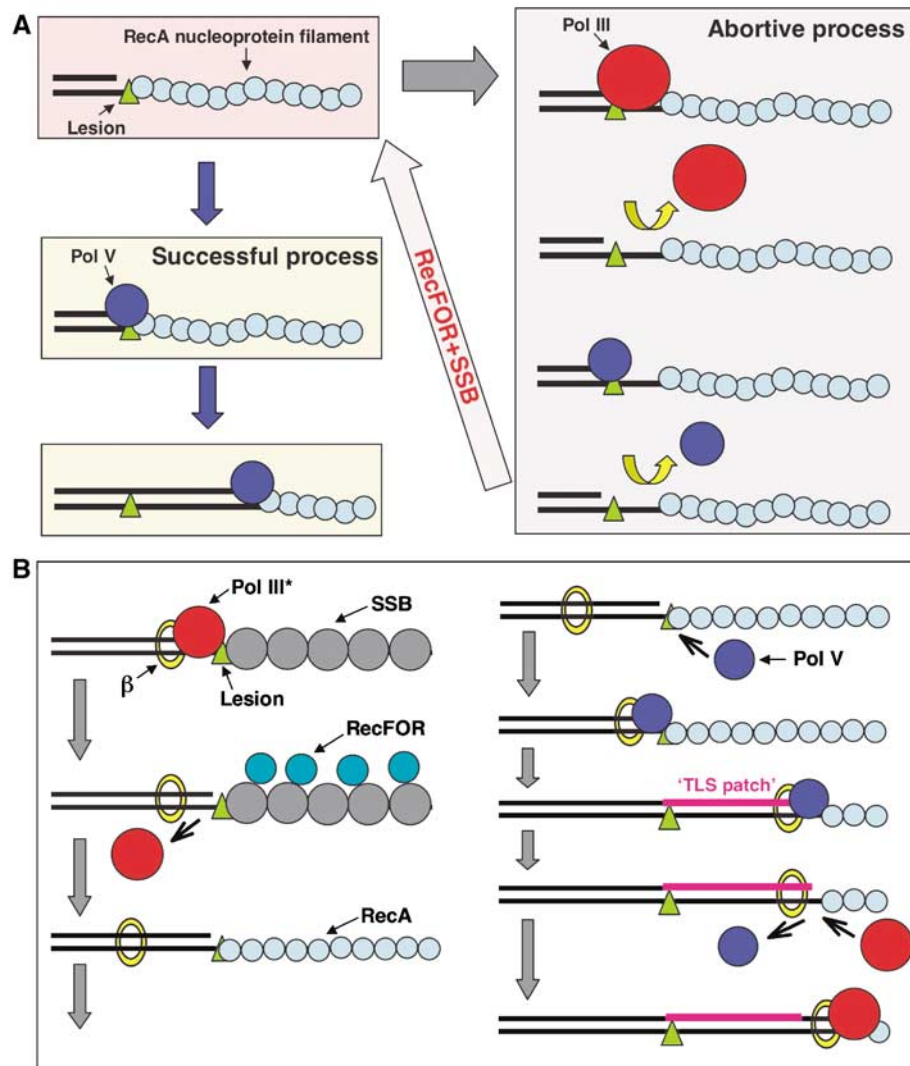


Figure 6 Model for TLS. **(A)** Interference between Pol III and Pol V. The results presented in this paper suggest that when a RecA/ATP filament is formed along the single-stranded DNA located downstream of a replication blocking lesion, the 3'-OH end of the primer can bind either Pol V or Pol III. When Pol V binds, TLS proceeds successfully as described previously (Fujii and Fuchs, 2004; Fujii *et al*, 2004). In contrast, binding of Pol III is not only an abortive event for the TLS reaction *per se*, but it also appears to locally perturb the structure of the 3'-tip of the RecA filament, thereby preventing successful binding of Pol V. Interestingly, our results show that both RecFOR and SSB proteins are able to rapidly 'reconstruct' the RecA filament for efficient Pol V-mediated lesion bypass, thus counteracting the adverse effect of Pol III on the bypass reaction. **(B)** Integrated TLS model: this scheme brings together the current data for the bypass of lesion by Pol V in *E. coli*. Upon reaching a replication block, Pol III dissociates leaving the beta-clamp on the template, while the replication fork helicase keeps moving, thus creating a region of single-stranded DNA downstream of the lesion (Higuchi *et al*, 2003; Pages and Fuchs, 2003; McInerney and O'Donnell, 2004). Owing to the higher affinity for ssDNA of SSB compared with RecA, SSB will bind first, thus preventing RecA filament formation. The RecFOR mediator proteins are essential for the formation of a RecA filament. Upon binding to the 3'-OH of the primer, Pol V will form a relatively stable initiation complex via a dual interaction with the beta-clamp and the tip of the RecA filament. Synthesis of a 'TLS patch' in excess of five nucleotides will allow Pol III to resume synthesis. We envision the process of TLS as a gap-filling reaction that occurs 'behind' the replication fork possibly involving free Pol III core rather than the Pol III holoenzyme (Lehmann and Fuchs, 2006).

propose an integrated model for lesion bypass (Figure 6B) that takes into account all known genetic requirements for induced mutagenesis. In *E. coli*, induced mutagenesis depends upon several key gene products (for recent reviews, see Goodman, 2002; Fuchs *et al*, 2004; Friedberg *et al*, 2005), namely *umuDC* (Kato *et al*, 1977; Steinborn, 1978) encoding Pol V, a Y-family polymerase responsible for the bypass of most lesions, *dnaN*, encoding the beta-clamp the general replication processivity factor (Becherel *et al*, 2002) and *recA* (Dutreix *et al*, 1989; Sweasy *et al*, 1990; Bailone *et al*, 1991). In addition to these factors, this paper illustrates the key function of *recF*, *recO* and *recR* genes in Pol V-mediated

lesion bypass. We specifically show that the defect in Pol V-mediated lesion bypass in *recF*, *recO* and *recR* strains is not due to the known delay in SOS induction kinetics in these strains (Hegde *et al*, 1995; Whitby and Lloyd, 1995). Indeed, the role of RecFOR proteins in Pol V-mediated lesion bypass directly hinges on the capacity of these proteins to load a RecA filament on single-stranded DNA coated by SSB. These RecA-covered regions appear to represent common intermediates for two alternative gap-filling pathways, namely Pol V-mediated TLS or *recF*-mediated recombination. Further experiments are required to shed light on the mechanisms that control the respective use of these two pathways. In

eukaryotes, although the direct role of a recombination filament has not yet been proven to be essential in lesion bypass, it was recently reported that RAD51 interacts with Pol eta and stimulates Pol eta-mediated D-loop extension (McIlwraith *et al*, 2005).

Materials and methods

Strains and mutation assays

Strains. Isogenic *recF*, *recO* and *recR* strains were constructed in the MGZ strain background derived from MG1655 (Napolitano *et al*, 2000) by P1 transduction using the following donor strains: [*recF400::Tn5*] from strain JJC450, [*recD1009 recO::Tn5*] from strain JJC404 and [*recR::Tn5*] from strain JJC1193. The following concentrations of antibiotics were used: 10 µg/ml tetracycline and 20 µg/ml kanamycin.

Survival curves. UV survival curves were established starting from fresh overnight cultures diluted 1/50 and grown under aeration to OD₇₀₀ = 0.5. Cells were pelleted and resuspended in 0.5 volume of 10 mM MgSO₄. Aliquots of 10 ml were irradiated with 254 nm UV light at doses ranging from 0 to 60 J/m². Adequate dilutions were plated on LB medium covered by aluminum foil to prevent potential photoreactivation.

Rifampicin resistance mutation assay

Cell irradiated at various UV doses were inoculated in LB medium (1/20 dilution) and shaken for 4 h at 37°C (0.5 ml suspended cells + 9.5 ml LB). Following this period, the cultures were centrifuged, resuspended and plated on either rifampicin plates (2–4 plates, rif = 100 µg/ml) or LB plates for determining the mutant and surviving fractions, respectively.

Single-adduct plasmid mutation assay

The different strains, the wild-type MGZ strain and the corresponding *recF*, *recO* and *recR* mutant strains were transformed with the low-copy number plasmid expressing Pol V from its wild-type promoter pRW134 (= O⁺-*umuD'*C) or the corresponding empty vector (pGB2). The corresponding strains were subsequently transformed with pCU-derived plasmids containing a single TT(6-4) photoproduct (Becherel and Fuchs, 1999) or the corresponding lesion-free plasmid and plated on indicator plates including ampicillin (Ap), spectinomycin (Sp), IPTG (0.3 mM) and X-gal (60 µg/ml). The photoproduct is located close to the 5'-end of the *LacZ* (alpha) gene. As the 3'-T of the photoproduct is part of an

in-frame TAA ochre codon, mutagenic bypass can be monitored directly on these plates as a change in colony color from white to blue. Indeed, the most frequent mutagenic bypass event of the 6-4 photoproduct, a T→C transition at 3'-T, converts the ochre codon into a sense codon (Becherel and Fuchs, 1999).

Proteins

Pol III*, Pol V, the beta-clamp and SSB were prepared as described (Fujii and Fuchs, 2004; Fujii *et al*, 2004). RecA was obtained from Pharmacia. The gamma-complex clamp loader and RecFOR proteins were generous gifts from CS McHenry (University of Colorado, Denver) and from KJ Marians (Memorial Sloan-Kettering Cancer Center, NY), respectively. DNA substrates are described in the figure legends and elsewhere (Fujii and Fuchs, 2004; Fujii *et al*, 2004).

DNA replication assays

The components of reaction buffer were 20 mM Tris-HCl (pH 7.5), 4% glycerol, 8 mM DTT, 80 µg/ml BSA, 2.5 mM ATP, 8 mM MgCl₂ and 0.1 mM each dNTP (dA, dT, dG and dC). The standard reaction was carried out as follows: 300 nM SSB (as a tetramer), 50 nM beta-clamp (as a dimer) and 2 nM primed template DNA were mixed and incubated for 5 min at 30°C, followed by the addition of 2 µM RecA. After 3 min, 0.1 µM RecF, 0.2 µM RecO and 10 µM RecR were mixed and incubated for 5 min at 30°C. The reaction was initiated by the addition of 62.7 nM Pol III*. After 3 min, 100 nM Pol V was mixed and incubated for 10 min at 30°C. The reaction was terminated by adding EDTA (the final concentration was 25 mM). Thereafter, the products were purified by phenol/chloroform extraction, followed by ethanol precipitation and EcoRI digestion. The reactions were stopped by adding 1 volume of formamide containing 25 mM EDTA and bromophenol blue. The products were heat denatured, separated on a 10% denaturing PAGE and visualized on a Molecular Imager (Bio-Rad). The percentage of TLS was calculated as follows: in the presence of Pol III and Pol V, TLS0 = intensity of TLS0 band/sum of TLS0 + L-1 + L0; with Pol V alone, TLS0 = sum of all bands above L0/sum of all bands above L-8.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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