The recombination mediator proteins RecFOR maintain RecA* levels for maximal DNA polymerase V Mut activity

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DNA template damage can potentially block DNA replication. Cells have therefore developed different strategies to repair template lesions. Activation of the bacterial lesion bypass DNA polymerase V (Pol V) requires both the cleavage of the UmuD subunit to UmuD’ and the acquisition of a monomer of activated RecA recombinase, forming Pol V Mut. Both of these events are mediated by the generation of RecA* via the formation of a RecA-ssDNA filament during the SOS response. Formation of RecA* is itself modulated by competition with the ssDNA-binding protein (SSB) for binding to ssDNA. Previous observations have demonstrated that RecA filament formation on SSB-coated DNA can be favored in the presence of the recombination mediator proteins RecF, RecO, and RecR. We show here using purified proteins that in the presence of SSB and RecA, a stable RecA-ssDNA filament is not formed, although sufficient RecA* is generated to support some activation of Pol V. The presence of RecFOR increased RecA* generation and allowed Pol V to synthesize longer DNA products and to elongate from an unpaired primer terminus opposite template damage, also without the generation of a stable RecA–ssDNA filament.

DNA template damage represents a potential block to DNA replication. As such, the cell has developed many different strategies to maintain genomic integrity in the face of template lesions. Overall, these DNA damage repair strategies divide into those that are error-free and those that are error-prone. The latter pathways reflect the action of specialized DNA polymerases that can bypass directly the lesions in the DNA template. *Escherichia coli* has three translesion synthesis (TLS)2 DNA polymerases: DNA polymerase (Pol) II, Pol IV (DinB (1)), and Pol V (UmuD’-UmuC (2, 3)). All three of these TLS polymerases are induced during the SOS response (4) and have been shown to be capable of lesion-induced or targeted mutagenesis (5). However, it is Pol V that is the prime agent of UV-induced mutagenesis discovered by Witkin (6).

UV-induced mutagenesis reflects an increase in spontaneous mutagenesis as a result of UV irradiation. Early models suggested that the replicative polymerase, the DNA polymerase III holoenzyme (7), might be modified by the action of the *umu* gene products to allow it to bypass template damage (8). Mutations in *umuD* and *umuC* eliminated the bulk of UV-induced mutagenesis (9, 10). However, it was demonstrated subsequently that UmuC itself possessed DNA polymerase activity and that the UmuD’-UmuC complex could bypass template damage in the presence of activated RecA (RecA*) (2, 3).

The TLS form of PolV contains a stoichiometric RecA monomer and has been termed a mutasome or Pol V Mut (11, 12) that must bind an ATP moiety to be active (13). Also, whereas it is clear that the RecA is transferred from the 3’ end of a RecA–single-stranded (ss) DNA filament to Pol V (14), there is some debate about whether the RecA filament has to be directly adjacent to and downstream of the template damage (i.e. in cis (15, 16) or is in trans on a ssDNA not associated with the damaged template (17, 18). RecA* is formed when ssDNA is generated as a result of replication forks encountering template lesions, as during the SOS response (19). The likely disposition of the replicated sister chromosomes is that of gaps in both nascent DNA strands (20) generated by the replisome skipping over the template lesion and continuing replication downstream (21, 22). It has been accepted that RecA must compete with SSB for binding to the ssDNA in the gaps to form a RecA–ssDNA filament. Biochemically, the recombination mediator proteins RecF, RecO, and RecR are known to facilitate RecA nucleation on SSB–ssDNA (23) and in gaps (24). It is not surprising, then, that recFOR mutants display a delay in the induction of the SOS response (25, 26).

These observations led Fujii et al. (27) to examine the role of the RecFOR proteins in TLS by Pol V. They found, using a primer extension assay, that TLS by Pol V in the presence of SSB, RecA, and the β sliding clamp required the RecFOR proteins. They suggested that the RecFOR requirement was a result of mediating RecA filament formation on the ssDNA. We have found a similar requirement for RecFOR in Pol V TLS and show that this requirement is an effect neither on access of Pol V to the 3’ end of the primer nor on Pol V DNA polymerase activity.
per se but reflects a demand to maintain RecA* levels sufficient for maximal Pol V Mut activity during lesion bypass.

Results and discussion
Reconstitution of TLS by Pol V

To examine Pol V TLS, we used the primer template (p/t) shown in Fig. 1A. Here the bottom, damage-containing template strand is 139 nt long. A 5'-32P] 27-nt-long primer is annealed such that its 3'-end is 5 nt upstream of the first thymidine residue in a cyclopyrimidine dimer (CPD) or 7 nt upstream of a tetrahydrofuran abasic site analog (THF). The ss template upstream of the 5'-end of the primer prevents the β processivity clamp from sliding off the primer template once it has been loaded.

DNA synthesis by Pol V was reconstituted in the presence of β, the DnaX clamp-loading complex (DnaX cx, τγδθ'ψ) (7), SSB, RecA, and RecFOR. The incubations were conducted in two steps. First, SSB, RecA, β, DnaX cx, RecA, and RecFOR were incubated with the p/t for 5 min at 37 °C. Pol V was then added, and the incubation continued for 8 min. Reaction products were recovered by ethanol precipitation after phenol-CHCl3 extraction, resuspended in denaturing loading dye, and electrophoresed through polyacrylamide gels (20%) containing 7 M urea. Product formation was quantified by phosphorimag-
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Fig. 1B shows the results of omitting each of the protein components individually from the reaction using each of the three templates (undamaged, CPD, and THF). Only Pol V and RecA were absolutely required for DNA synthesis (Fig. 1B, left panel, compare lanes 2 and 5 with lane 1), β and SSB stimulated the reaction slightly (by ~50%, Fig. 1B, left panel, compare lanes 3 and 4 with lane 1), and the presence of RecFOR activated slightly (Fig. 1B, left panel, compare lane 9 with lane 1). The absence of RecR was strongly inhibitory, indicating that the presence of RecR modulates the activities of RecF and RecO, as has been observed previously (23, 28, 29) (Fig. 1B, left panel, compare lane 8 with lane 1). Thus, RecFOR are not required for Pol V-catalyzed DNA polymerase activity, nor are they required to displace SSB from the ssDNA to facilitate formation of a RecA–ssDNA filament to generate RecA*. The equilibrium between SSB and RecA binding to the template DNA presumably allows sufficient RecA* formation to support Pol V activity. Pol V appeared to be capable of polymerizing a limited number of nucleotides on ssDNA coated with RecA (Fig. 1B, left panel, compare lane 4 with lane 1), although this reaction is likely related to the stability of the RecA filament near the primer terminus. However, the addition of RecFOR had a distinct effect on the distribution of the DNA products in the reaction (Fig. 1B, left panel, compare lane 1 with lane 9). The DNA products were longer in the presence of RecFOR (Fig. 1C), suggesting that Pol V processivity either had increased or had a greater level of RecA* than could be achieved in the presence of RecA and SSB was required for extensive Pol V-catalyzed DNA synthesis.

Note that the complete lack of DNA synthesis in the absence of RecA (Fig. 1B, left panel, lane 5) indicates that no other component of the reaction was contaminated to any substantive extent with a DNA polymerase activity. Similarly, the lack of DNA synthesis activity in the absence of Pol V (Fig. 1B, left panel, lane 1), although this reaction is likely related to the stability of the RecA filament near the primer terminus. However, the addition of RecFOR had a distinct effect on the distribution of the DNA products in the reaction (Fig. 1B, left panel, compare lane 1 with lane 9). The DNA products were longer in the presence of RecFOR (Fig. 1C), suggesting that Pol V processivity either had increased or had a greater level of RecA* than could be achieved in the presence of RecA and SSB was required for extensive Pol V-catalyzed DNA synthesis.

The results were different when damage-containing p/ts were used (Fig. 1B, center and right panels). Interestingly, β and SSB were not required for TLS but were required for extension from the damage (Fig. 1B, center and right panels, compare lanes 3 and 4). The requirement of β for bypass has been noted previously (2, 15, 30), although Pol V activity was not parsed in the same manner as we do here (i.e. TLS and extension). Indeed, Maor-Shoshani and Livneh (31) noted that β did not stimulate initiation of DNA synthesis by Pol V but did stimulate bypass. Examination of their data suggests that the stimulation of bypass they observed was similar to what we observe here, i.e. stimulation of extension past the damage.

RecFOR stimulated extension past the damage on both the CPD and THF templates, whereas they had little effect on either TLS or overall DNA polymerase activity (Fig. 1B, center and right panels, compare lanes 1 and 9). We reasoned that this apparent requirement for RecFOR in extension from damage and the apparent increase in processivity observed in the presence of RecFOR on the undamaged template might both reflect the action of RecFOR in generating RecA*. If this is the case, one would predict that the RecFOR effect would depend on the presence of SSB in the reaction.

Effect of SSB on RecFOR stimulation of Pol V activity

RecFOR are sufficient to exchange RecA with SSB on SSB-coated ssDNA (23), and RecF is thought to provide nucleation for this exchange at the double-stranded edges of gaps (24). We therefore examined the effect of RecOR and RecFOR on Pol V activity in the presence and absence of SSB on the three templates (Fig. 2). Comparison of the extents of elongation on the various templates showed that RecFOR stimulated extension on the CPD and THF templates by roughly 5- and 6-fold, respectively, whereas there was little effect in the absence of SSB (Fig. 2B). RecOR had little effect in either the presence or absence of SSB with the CPD template but did show approximately half the stimulatory effect as RecFOR in the presence of SSB with the THF template (Fig. 2B). The requirement for RecF for the maximum effect is interesting and not obviously explained by current biochemistry. These templates do not model a gap in newly replicated DNA because there is no 5′-ended DNA downstream of the template damage where one might expect RecF to facilitate 5′ → 3′ RecA filament growth.
toward the primer terminus. On the other hand, RecF, with RecR, has been shown to limit extension of the RecA filament from ssDNA onto dsDNA (32). Extension of the RecA filament at the primer terminus onto the dsDNA formed by the primer and the template would presumably inhibit DNA synthesis by Pol V; thus, preventing such extension could result in the stimulation we observe.

It is difficult to judge the dynamic between SSB and RecA with the Pol V system because of the absolute requirement for RecA to observe polymerase activity. We therefore turned to another Y family TLS polymerase from *E. coli*, DNA polymerase IV, and used it to replace Pol V in the primer extension assay on an undamaged template (Fig. 3). SSB stimulated extension by DNA Pol IV (Fig. 3, compare lanes 1 and 2), whereas RecA alone inhibited extension (Fig. 3, compare lanes 4 and 2), although it did not shut it down completely, suggesting that the region at the 3′ end of the RecA–ssDNA filament is dynamic, allowing some access by the polymerase. SSB alone was sufficient to overcome the RecA inhibition (Fig. 3, compare lanes 3 and 4), arguing that under our conditions, even though RecA is in 20-fold molar excess of SSB tetramers, the preferred agent binding to the ssDNA is SSB and furthermore suggesting that mixed SSB–RecA–ssDNA filaments are fairly unstable. RecFOR alone had no significant effect (Fig. 3, compare lanes 2 and 7), suggesting that RecF was not competing with Pol IV for the primer terminus (it does bind both ends of a gap (24)) and that RecO, which is presumably bound to the ssDNA template, does not inhibit polymerization. Even in the presence of all five proteins, SSB, RecA, and RecFOR, Pol IV extended most of the primer to full length (Fig. 3, lane 6). Thus, the presence of RecFOR does not result in the generation of a stable RecA–ssDNA filament under these conditions, where we would expect to observe inhibition (as in Fig. 3, lane 4). If RecFOR are displacing SSB from the DNA to load RecA, SSB binding to ssDNA, RecA–ssDNA filament formation and dissociation, and SSB competition with RecA for binding to ssDNA are thus likely to be quite dynamic.

Another assay for examining whether a stable RecA–ssDNA filament was forming was developed by Arad et al. (33). These authors showed that accessibility of the 3′ end of the primer, as measured by the ability of exonuclease III (Exo III) to degrade it, was inhibited by RecA but favored by SSB. Using this assay (Fig. 4), RecA inhibited Exo III digestion of the primer (Fig. 4, compare lanes 1 and 2), as expected. SSB had little effect on its own (Fig. 4, compare lanes 1 and 3), as did the loading of β (Fig. 4, compare lanes 1 and 4), suggesting that Exo III could push the β clamp back toward the 5′ end of the primer as it digested the DNA. Exo III digestion was partially recovered when RecA and SSB were present together (Fig. 4, compare lanes 1, 2, 3, and 7), consistent with our conclusions above that SSB was sufficient to displace (or exclude) RecA from the ssDNA template. In the presence of RecA and SSB, the addition of different combinations of RecF, O, and R did not result in any significant increased inhibition of digestion, compared with the combination of RecA and SSB (Fig. 4, compare lanes 7 to lanes 8–14), confirming that under the conditions of our assay, RecFOR was not establishing a stable RecA–ssDNA filament.

RecFOR stimulation of Pol V elongation past template damage is bypassed by RecA730

Our data suggest that in the presence of SSB and RecA, RecFOR are not required to form PolV Mut that is capable of sub-
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substantial DNA synthesis on undamaged templates, although the length distribution of DNA products increases in the presence of RecFOR. However, stimulation by RecFOR becomes apparent when Pol V faces the difficult task of elongating from the unpaired primer terminus opposite a site of template damage. We propose that the RecFOR requirement for the latter reflects the need for an increased level of RecA* required for Pol V Mut to maintain activity. Such a model would also account for the increase in DNA synthesis patch length on the undamaged templates and is consistent with the requirement for the presence of SSB to observe any RecFOR effect. If the model is correct, we would expect that the RecFOR requirement would be bypassed with a variant RecA that was capable of constitutively generating RecA*. Such a variant is RecA730.

RecA730 was derived from RecA441 (34), a recA mutant initially known as tif-1, which was constitutively induced for prophage induction at elevated temperature (35). RecA730 was shown, unlike WT RecA, to be able to displace SSB from ssDNA at low (1 mM) concentrations of magnesium and at all temperatures tested (36), an ability that was attributed to a more rapid association with ssDNA than the WT. Constitutive induction of the SOS response was therefore attributed to the assembly of sufficient RecA730 patches on ssDNA regions that are typically available in an unstressed cell.

The activity of WT RecA and RecA730 was compared on the THF p/t in the presence of RecOR or RecFOR or in their absence (Fig. 5). RecA730 displayed the same effect of increasing the length distribution of the DNA synthesis products as we observed previously with RecFOR and WT RecA (Fig. 5A, compare lanes 6 and 1 with lane 5, and B). RecA730 stimulated elongation past the damage in the absence of RecFOR to a greater extent than observed with WT RecA in the presence of RecFOR (Fig. 5A, compare lanes 6 and 1). The nearly identical behavior of RecA730 compared with WT RecA plus RecFOR indicates that the observed effects of RecFOR reported above relate to the necessity of maintaining a sufficient pool of RecA* that will add the necessary activated RecA monomer to Pol V to form Pol V Mut.

Suppression of LexA cleavage in the presence of RecA and SSB indicates limited RecA filament formation

Whereas our Pol V assays require the transfer of an activated monomer of RecA* from the RecA–ssDNA filament to Pol V, to support our argument that stable RecA–ssDNA filaments were not forming, we used another assay that more directly assesses formation of RecA–ssDNA filaments. Cleavage of the SOS repressor LexA requires binding directly to the deep helical groove of the RecA–ssDNA filament (37, 38). To measure LexA cleavage, we purified a modified version of LexA that was developed by John Little (39) in which a portion of the LexA N terminus was deleted and replaced with His and protein kinase A tags (AN–His–PKA–LexA (NHP–LexA)). NHP–LexA was shown to be cleaved in the presence of RecA–ssDNA filaments at the same rate as full-length, WT LexA (39).

We examined [32P]NHP-LexA cleavage under conditions identical to those of the Pol V primer extension assay using either RecA or RecA730. RecA730-mediated NHP-LexA cleavage in the absence of SSB was ~50% greater than that in the presence of RecA (Fig. 6, compare lanes 3 and 7), similar to what has been reported previously (36), and, as expected, based on our data presented above, RecA-mediated cleavage of NHP-LexA was almost completely inhibited in the presence of SSB (Fig. 6, compare lanes 3 and 4), supporting our conclusion that in the presence of SSB and RecA, a stable RecA–ssDNA filament is not formed. Surprisingly, SSB also inhibited RecA730-mediated LexA cleavage significantly, although there was, unlike with RecA, detectable residual cleavage (Fig. 6, compare lanes 7 and 8). The presence of either RecOR or RecFOR led to a partial recovery of LexA cleavage in the presence of SSB with both RecA and RecA730 (Fig. 6, compare lanes 5 and 6 with lanes 4 and lanes 9 and 10 with lane 8).

Because, unlike with RecA, RecFOR had little effect on RecA730-supported Pol V activity in the presence of SSB (Fig. 5), we suggest that the difference in LexA cleavage mediated by RecA730 compared with RecA in the presence of SSB reports on the extent of RecA* generation required for maintenance of maximal Pol V activity. Thus, using two different assays, one
that measures the generation of activated monomers of RecA and one that measures the generation of RecA–ssDNA filaments more directly, we show that stable RecA filaments do not form in the presence of SSB and either RecA or RecA 730 under the conditions described.

We note our surprising result that RecA730-ssDNA filament formation was inhibited by SSB similar to RecA–ssDNA filament formation as being inconsistent with a previous report (40). Whereas we cannot explain the different results for certain, one possible explanation might lie in the fact that in the previous report (36) M13 phage DNA was used as the DNA effector. Whereas this DNA is often used as a ssDNA, it is, in fact, highly structured (41) with extensive double-stranded regions. Therefore it is possible that Lavery and Kowalczykowski (36) were reporting on results where both RecA–ssDNA and RecA dsDNA filaments were forming. The latter can also mediate LexA cleavage (42).

Conclusions

As judged by the observations that RecA in the absence of SSB strongly inhibits Pol V DNA synthesis activity (Fig. 1) and that this inhibition is obviated when SSB is present, we argue that even in the presence of RecFOR, a stable, exclusively RecA–ssDNA filament does not form. This conclusion is strongly supported by similar results measuring LexA cleavage (Fig. 6), an assay that more directly assesses RecA–ssDNA filament formation. This view is somewhat counter to the established view that at elevated magnesium concentrations RecA can displace SSB (43). Nevertheless, RecA* is clearly being formed in our primer extension assays because Pol V DNA synthesis is activated. We suggest that the more likely scenario is that formation and dissociation of the RecA–ssDNA filament in the presence of SSB is quite dynamic, presumably mediated by mixed SSB–RecA–ssDNA filaments. This equilibrium results in the generation of sufficient Pol V Mut for simple elongation of a primer terminus. However, more difficult synthesis, for example, extensive synthesis of long DNA products with the undamaged template and extension from a primer terminus opposite a site of template damage, requires either higher concentrations of or continuous production of RecA*. It is therefore in these scenarios that we find a significant stimulation of Pol V Mut activity by the RecA mediator proteins RecFOR. The underlying reason for what we have observed is that Pol V Mut processivity is likely to be very low, and the polymerase may require repeated attempts before it is successful in elongating from an unpaired primer terminus.

Pol V Mut has been shown to possess an intrinsic ATPase activity that governs binding of the polymerase to the p/t (13). Binding to the p/t requires the nucleotide cofactor, whereas its hydrolysis favors dissociation of the polymerase. Turnover of ATP, compared with that of the nonhydrolyzable analog ATPγS is very rapid and very little DNA synthesis or TLS activity was evident in the presence of ATP. In fact, bypass of a THF in the presence of transactivated Pol V Mut required the presence of RecA*. These observations are consistent with what we have reported herein.

We therefore speculate that the inherent activity of Pol V Mut is likely to be very low, possibly even only one nucleotide incorporation event per association with one RecA monomer. Such an activity cycle would act to keep the patch size synthesized by Pol V quite small and would provide a feedback loop; in the case of SOS induced by UV irradiation, for example, ss gaps that accumulate because of the replisome skipping over the lesions are the likely source of RecA–ssDNA filament formation (19, 21, 44). As those gaps are filled in during repair, the level of RecA* available will decline rapidly, and Pol V Mut activity, if stochiometrically dependent on association with an activated RecA monomer and ATP, will therefore also do the same.

Experimental procedures

DNA template

An linear DNA template (139-mer) was constructed using oligonucleotides containing a single THF or a CPD adduct. The templates were constructed by ligating the following three oligonucleotides: PR1, a 59-mer (5′-TGT GAG CGG ATA ACA ATT TCA CAC AGG AAA CAG CTA TGA CCA TTA CGA GAG TCT AA-3′); PR2 (undamaged, CPD, or THF), a 38-mer (5′-ACG CGC TCT GCT AAC ATA CTT CGT ATT GAG TCT GAG GAG GAG GAG GAG GAG TCT AA-3′), where the underlining indicates the site of the template damage; and PR3, a 42-mer (5′-CGC ACC ACT TGG CAC TGG CCG TCG TTT TAC AAC GTC GTG ACT-3′) in the presence of a scaffold 84-mer, and PR4 (5′-CGA CGG CCA GTG CCA AGT GGT GGG TTA GAC TCA ATA CGA AGT ATG TTA GCA GAC AGC GTG ACT GAA TTC GTA ATC ATG ATC AT-3′). PR2 and PR3 were phosphorylated using ATP and bacteriophage T4 polynucleotide kinase (NEB) in 1× bacteriophage T4 DNA ligase buffer (NEB). PR1 (100 pmol), 5′-pPR2 (100 pmol), 5′-pPR3 (100 pmol), and PR4 (200 pmol) were annealed in 50 μl of 10 mM Tris-HCl (pH 7.5 at 25 °C), 1 mM EDTA, and 50 mM NAcI by heating to 95 °C and cooling slowly to room temperature. The mixture of annealed oligonucleotides was then treated with bacteriophage T4 DNA ligase (NEB) in 60 μl of 1× ligase buffer for 16 h at 16 °C. The DNA products were then denatured in 1× formamide loading buffer (40% formamide, 5 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue) and electrophoresed through a 10% (19:1 acrylamide to bisacrylamide) polycryl-
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amidine gel containing 7 M urea using 100 mM Tris borate (pH 8.3), 2 mM EDTA as the electrophoresis buffer. The 139-mer DNA template band was located by UV shadowing and eluted by crush and soak in 10 mM Tris-HCl (pH 8.0 at 25 °C), 1 mM EDTA for 16 h at 37 °C. The slurry was centrifuged through a Spin-X centrifuge tube filter (Corning), and the eluted DNA was recovered by ethanol precipitation. The polyacrylamide gel-purified primer PR5 (5′-GTG CGT TAG ACT CCT CAA TAC GAA GTA-3′) was 5′ end-labeled with [32P] using T4 kinase and [γ-32P]ATP as above. Excess ATP was removed by purification through a Sephadex G-50 spin column (GE Healthcare), and the labeled primer was annealed to the template as above.

Enzymes

His-tagged Pol V was purified as described (45). DnaX complex was the gift of Charles McHenry (University of Colorado) (46). SSB (47), β (48), RecA (49), RecF (49), RecO (49), RecR (49), and DNA polymerase IV (50) were lab stocks. RecA730 (51) was the gift of Michael Cox (University of Wisconsin). Exonuclease III was from NEB. ΔN-His-PKALexA was purified from BL21pLysS(pJWL944). The cells were grown in LB medium to A600 = 0.4, induced with 0.4 mM isopropyl-β-thiogalactopyranoside for 3 h, harvested, resuspended in 50 mM Tris-HCl (pH 7.5 at 4 °C), 10% sucrose, frozen in liquid N2, and stored at −80 °C. Cell suspension (21 g) was thawed and diluted with 180 ml of buffer 1 (25 mM Tris-HCl (pH 7.5 at 4 °C), 500 mM NaCl) + 10 mM imidazole HCl (pH 7.5); the cells were lysed by sonication, and insoluble material was cleared by centrifugation in a Savarll SLA1500 rotor at 13,000 rpm for 1 h. The supernatant (fraction 1, 190 ml, 722 mg) was applied to a 4-ml nickel–nitrilotriacetic acid column equilibrated in buffer 1 + 25 mM imidazole-HCl. The column was washed with 12 ml of equilibration buffer, 30 ml of buffer 1 + 50 mM imidazole HCl and eluted with a 40-ml gradient of 50–500 mM imidazole HCl in buffer 1. Fractions containing NHP-LexA were located by SDS-PAGE and pooled to give fraction 2 (14.5 ml, 110 mg). Fraction 2 (4 ml) was dialyzed against buffer 2 (200 mM NaCl, 0.1 mM EDTA, 10% glycerol) + 25 mM KPi (pH 7.0) and applied to a 13-ml hydroxyapatite column equilibrated with the same buffer. The column was washed with 30 ml of equilibration buffer and eluted with a 130-ml gradient of 25–400 mM KPi in buffer 2. Fractions containing NHP-LexA were located by SDS-PAGE, pooled, dialyzed against storage buffer (50 mM imidazole HCl, pH 7.1, 1 mM EDTA, 200 mM NaCl, 35% glycerol) to give fraction 3 (5 ml, 13.4 mg), aliquoted, frozen in liquid N2, and stored at −80 °C. Plasmid pJWL944 was the gift of John Little (University of Arizona).

 Primer extension assay

Standard reaction mixtures (5 μl) contained 20 mM Tris-HCl (pH 7.5), 4% (v/v) glycerol, 8 mM DTT, 80 μg/ml BSA, 3 mM ATP, 8 mM MgCl2, and 0.5 mM dNTPs. The standard reaction was carried out as follows: 100 nM SSB (tetramer), 50 nM β (dimer), 5 nM DnaX cx (τ2γδδ’χψ), 2 μM RecA, and when indicated, 50 nM RecF, 0.5 μM RecO, and 0.5 μM RecR were first incubated with 2 nM p/t for 5 min at 37 °C. DNA synthesis was initiated by the addition of 500 nM DNA Pol V, and the reactions were incubated for 8 min at 37 °C. The reactions were terminated by adding 30 mM EDTA. The products were then purified in the presence of 10 ng of tRNA as carrier by phenol/chloroform extraction followed by ethanol precipitation and resuspended in formamide loading buffer. After heat denaturation at 95 °C for 5 min, the products were separated by electrophoresis through a 20% polyacrylamide gel (19:1 acrylamide to bisacrylamide) containing 7 M urea using 100 mM Tris borate (pH 8.3), 2 mM EDTA as the electrophoresis buffer. The gels were fixed by soaking in 10% methanol, 7% HOAc, 5% glycerol and dried. The dried gels were autoradiographed, and DNA products were quantified using phosphorimaging and ImageGauge software (Fuji). In reactions with Pol IV, the polymerase was present at 20 nM, and the incubation was for 4 min.

 Primer accessibility assay

Reaction conditions were as for the primer extension assay except that dNTPs and Pol V were omitted and E. coli Exo III (0.005 unit, NEB) was included. All other proteins were at the same concentrations as in the primer extension assay. The reactions were carried out at 37 °C as follows. RecA and the DNA substrate were first incubated for 2 min. Next SSB, β, DnaX cx, and RecFOR proteins were added as indicated, and the reactions were further incubated for 4 min. Exo III was then added, and the incubation was continued for 4 min. The reaction products were recovered and analyzed as for the primer extension assay.

 LexA cleavage assay

NHP-LexA (100 μM) was labeled in a 20-μl reaction mixture containing 1× PKA buffer (NEB), 200 μM ATP, 30 μCi of [γ-32P]ATP, and 2500 units of protein kinase A (NEB) for 45 min at 30 °C. The reaction was terminated by the addition of EDTA to 50 mM, and [32P]NHP-LexA was separated from residual ATP by gel filtration through a Biogel P10 column (1 ml) equilibrated and developed in NHP-LexA storage buffer. The [32P]NHP-LexA pool was stored at −20 °C. LexA cleavage reaction mixtures were identical to primer extension reaction mixtures for Pol V and contained β, DnaX cx, p/t, 2 μM NHP-LexA, ~5,000 Cerenkov cpm of [32P]NHP-LexA and RecA, RecA730, SSB, and either RecOR or Rec FOR as indicated. The reactions were incubated for 13 min, mixed with an equal volume of 2× SDS loading dye, and analyzed by 20% SDS-PAGE. The gels were fixed in 10% methanol, 7% HOAc, 5% glycerol; dried; and imaged by phosphorimaging and autoradiography. The gels were quantified using Fuji ImageGauge software.

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References

1. Wagner, J., Gruz, P., Kim, S. R., Yamada, M., Matsu, K., Fuchs, R. P., and Nohmi, T. (1999) The dinB gene encodes a novel E. coli DNA polymerase,
DNA pol IV, involved in mutagenesis. Mol. Cell 4, 281–286 CrossRef Medline

2. Tang, M., Shen, X., Frank, E. G., O’Donnell, M., Woodgate, R., and Goodman, M. F. (1999) UmuD’C is an error-prone DNA polymerase, Escherichia coli pol V. Proc. Natl. Acad. Sci. U.S.A. 96, 8919–8924 CrossRef Medline

3. Reuven, N. B., Arad, G., Maor-Shoshani, A., and Livneh, Z. (1999) The mutagenesis protein UmuC is a DNA polymerase activated by UmuD’, RecA, and SSB and is specialized for translesion replication. J. Biol. Chem. 274, 31763–31766 CrossRef Medline

4. Fijalkowska, I. J., Schaper, R. M., and Jonczyk, P. (2012) DNA replication fidelity in Escherichia coli: a multi-DNA polymerase affair. FEMS Microbiol. Rev. 36, 1105–1121 CrossRef Medline

5. Napolitano, R., Janel-Bintz, R., Wagner, J., and Fuchs, R. P. (2000) All three SOS-inducible DNA polymerases (Pol II, Pol IV and Pol V) are involved in induced mutagenesis. EMBO J. 19, 6259–6265 CrossRef Medline

6. Witkin, E. M. (1976) Ultraviolet mutagenesis and inductive DNA repair in Escherichia coli. Bacteriol. Rev. 40, 869–907 Medline

7. McHenry, C. S. (2011) DNA replicas from a bacterial perspective. Annu. Rev. Biochem. 80, 403–436 CrossRef Medline

8. Goodman, M. F., and Woodgate, R. (2013) Translesion DNA polymerases. Cold Spring Harb. Perspect. Biol. 5, a010363 CrossRef Medline

9. Kato, T., and Shinoura, Y. (1977) Isolation and characterization of mutants of Escherichia coli deficient in induction of mutations by ultraviolet light. Mol. Gen. Genet. 156, 121–131 Medline

10. Steinborn, G. (1978) Uvm mutants of Escherichia coli K12 deficient in UV mutagenesis: I. Isolation of uvm mutants and their phenotypic characterization in DNA repair and mutagenesis. Mol. Gen. Genet. 165, 87–93 CrossRef Medline

11. Schlacher, K., Leslie, K., Wyman, C., Woodgate, R., Cox, M. M., and Goodman, M. F. (2005) DNA polymerase V and RecA protein, a minimal mutasome. Mol. Cell 17, 561–572 CrossRef Medline

12. Jiang, Q., Karata, K., Woodgate, R., Cox, M. M., and Goodman, M. F. (2009) The active form of DNA polymerase V is UmuD’C-RecA-ATP. Nature 460, 359–363 CrossRef Medline

13. Erdem, A. L., Jaszcuz, M., Bertram, J. G., Woodgate, R., Cox, M. M., and Goodman, M. F. (2014) DNA polymerase V activity is autoregulated by a novel intrinsic DNA-dependent ATPase. eLife 3, e02384 CrossRef Medline

14. Dutreix, M., Moreau, P. L., Bailone, A., Galibert, F., Battista, J. R., Walker, G. C., and Devoret, R. (1989) New recA mutations that dissociate the various RecA protein activities in Escherichia coli provide evidence for an additional role for RecA protein in UV mutagenesis. J. Bacteriol. 171, 2415–2423 CrossRef Medline

15. Fujii, S., and Fuchs, R. P. (2009) Biochemical basis for the essential genetic requirements of RecA and the β-clamp in Pol V activation. Proc. Natl. Acad. Sci. U.S.A. 106, 14825–14830 CrossRef Medline

16. Fuchs, R. P., and Fujii, S. (2013) Translesion DNA synthesis and mutagenesis in prokaryotes. Cold Spring Harb. Perspect. Biol. 5, a012682 CrossRef Medline

17. Schlacher, K., Cox, M. M., Woodgate, R., and Goodman, M. F. (2006) RecA acts in trans to allow replication of damaged DNA by DNA polymerase V. Nature 442, 883–887 CrossRef Medline

18. Patel, M., Jiang, Q., Woodgate, R., Cox, M. M., and Goodman, M. F. (2010) A new model for SOS-induced mutagenesis: how RecA protein activates DNA polymerase V. Crit. Rev. Biochem. Mol. Biol. 45, 171–184 CrossRef Medline

19. Sassanfar, M., and Roberts, J. W. (1990) Nature of the SOS-inducing signal in Escherichia coli: the involvement of DNA replication. J. Biol. Chem. 212, 79–96 CrossRef Medline

20. Rupp, W. D., and Howard-Flanders, P. (1968) Discontinuities in the DNA synthesized in an excision-defective strain of Escherichia coli following ultraviolet irradiation. J. Mol. Biol. 31, 291–304 CrossRef Medline

21. Yeeles, J. T., and Marins, K. J. (2011) The Escherichia coli replosome is inherently DNA damage tolerant. Science 334, 235–238 CrossRef Medline

22. Yeeles, J. T., Poli, J., Marins, K. J., and Pasero, P. (2013) Rescuing stalled or damaged replication forks. Cold Spring Harb. Perspect. Biol. 5, a012815 CrossRef Medline
42. Lu, C., and Echols, H. (1987) RecA protein and SOS. Correlation of mutagenesis phenotype with binding of mutant RecA proteins to duplex DNA and LexA cleavage. *J. Mol. Biol.* 196, 497–504 CrossRef Medline

43. Lavery, P. E., and Kowalczykowski, S. C. (1988) Biochemical basis of the temperature-inducible constitutive protease activity of the RecA441 protein of *Escherichia coli*. *J. Mol. Biol.* 203, 861–874 CrossRef Medline

44. Rupp, W. D., and Howard-Flanders, P. (2005) Discontinuities in the DNA synthesized in an excision-defective strain of *Escherichia coli* following ultraviolet irradiation 1968. *DNA Repair (Amst.)* 4, 620–633 Medline

45. Karata, K., Vaisman, A., Goodman, M. F., and Woodgate, R. (2012) Simple and efficient purification of *Escherichia coli* DNA polymerase V: cofactor requirements for optimal activity and processivity in vitro. *DNA Repair (Amst.)* 11, 431–440 CrossRef Medline

46. Pritchard, A. E., Dallmann, H. G., Glover, B. P., and McHenry, C. S. (2000) A novel assembly mechanism for the DNA polymerase III holoenzyme DnaX complex: association of δδ′ with DnaX(4) forms DnaX(3)δδ′. *EMBO J.* 19, 6536–6545 CrossRef Medline

47. Minden, J. S., and Marians, K. J. (1986) *Escherichia coli* topoisomerase I can segregate replicating pBR322 daughter DNA molecules in vitro. *J. Biol. Chem.* 261, 11906–11917 Medline

48. Johanson, K. O., Haynes, T. E., and McHenry, C. S. (1986) Chemical characterization and purification of the β subunit of the DNA polymerase III holoenzyme from an overproducing strain. *J. Biol. Chem.* 261, 11460–11465 Medline

49. Xu, L., and Marians, K. J. (2003) PriA mediates DNA replication pathway choice at recombination intermediates. *Mol. Cell* 11, 817–826 CrossRef Medline

50. Gabbai, C. B., Yeeles, J. T., and Marians, K. J. (2014) Replisome-mediated translesion synthesis and leading strand template lesion skipping are competing bypass mechanisms. *J. Biol. Chem.* 289, 32811–32823 CrossRef Medline

51. Eggler, A. L., Lusetti, S. L., and Cox, M. M. (2003) The C terminus of the *Escherichia coli* RecA protein modulates the DNA binding competition with single-stranded DNA-binding protein. *J. Biol. Chem.* 278, 16389–16396 CrossRef Medline