Biochemical basis for the essential genetic requirements of RecA and the β-clamp in Pol V activation

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In *Escherichia coli*, it is genetically well established that the β-clamp and RecA are essential cofactors that endow DNA polymerase (Pol) V with lesion bypass activity. However, the biochemical basis for these requirements is still largely unknown. Because the process of translesion synthesis (TLS) requires that the specialized DNA polymerase synthesize in a single binding event a TLS patch that is long enough to resist external proofreading, it is critical to monitor Pol V burst synthesis. Here, we dissect the distinct roles that RecA and the β-clamp perform during the Pol V activation process using physiologically relevant long single-stranded template DNA, similar to those used in genetic assays. Our data show that the β-clamp endows the complex between Pol V and the template DNA with increased stability. Also, the RecA filament formed in *cis* on the single-stranded DNA produced downstream from the lesion stretches the template DNA to allow smooth elongation of the nascent strand by Pol V. The concurrent action of both cofactors is required for achieving productive TLS events. The present article presents an integrated view of TLS under physiologically relevant conditions in *E. coli* that may represent a paradigm for lesion bypass in other organisms.

When a replication apparatus encounters a replication-blocking lesion in one of the template strands, the replicative helicase keeps opening the parental duplex, replication continues on the undamaged strand, and a gap is formed in the damaged chromatid opposite the lesion (1, 2). Functional uncoupling of the leading and lagging strand DNA polymerases has been observed both in vivo (3) and in vitro (4, 5). The single-stranded gaps formed opposite the lesions are converted into the molecular signal for the SOS response on formation of a single-stranded ssDNA-RecA filament. Translesion DNA synthesis (TLS) that is largely mediated by DNA polymerase (Pol) V (encoded by the *umuDC* operon) requires RecA (6–10) and the β-clamp, the replication processivity factor (11). In vivo, RecA filament formation is a complex reaction that involves the recombination mediator proteins RecFOR to counteract the high propensity of ssDNA-binding (SSB) protein to bind ssDNA (12). Indeed, RecFOR proteins are essential for efficient Pol V-mediated TLS and mutagenesis both in vitro and in vivo (13). Naked regions of ssDNA are unlikely to persist in vivo even for short time periods as shown during lagging-strand replication (14). Because Pol V-mediated TLS is induced late during the SOS response, the ssDNA gaps generated downstream the replication-blocking lesions are initially covered by SSB and gradually replaced by RecA in a RecFOR mediated reaction path. It is, thus, sensible to include these proteins in Pol V-mediated TLS reconstitution experiments as we showed previously (13). The mechanism by which RecA activates Pol V has been a matter of debate over the years (15), and we will briefly discuss it in the light of a recently published article (16).

TLS entails at least two DNA polymerase switches, first from Pol III holoenzyme (HE) to Pol V, and then back to Pol III. Whereas the first switch occurs at the lesion site as a consequence of the inability of Pol III to replicate past the lesion, the position of the second swap critically determines whether the overall bypass reaction is successful or not (17, 18). In vivo, lesion bypass is achieved under conditions where multiple DNA polymerases are present jointly, competing with each other (19). During TLS, the intermediate that is released on dissociation of Pol V becomes a substrate for another DNA polymerase, possibly Pol III or Pol II. Therefore, for a TLS event to be successful, it is essential that the TLS intermediate withstands degradation by proofreading exonucleases. Because Pol III and Pol II sense lesions located <4 or 5 bp away from the primer terminus, it is essential that the TLS patch size made by Pol V during a single binding event is larger than 5 nt (17). Thus, to analyze the biochemistry of Pol V, in a way that is biologically relevant to the overall process of TLS, it is essential to implement experiments under single-turnover conditions. In the present article, we present evidence that a *cis*-RecA filament and the β-clamp are necessary and sufficient to support TLS under physiologically relevant conditions.

Results and Discussion

Pol V Forms a Stable Initiation Complex on Interaction with the β-Clamp and *cis*-RecA. For the reasons outlined above, we wanted to measure the intrinsic processivity of Pol V in the absence of DNA damage. We were particularly concerned by the debate over the nature of the RecA filament that is required for Pol V activity. Although in our previous work we have shown efficient activation of Pol V for TLS by a *cis*-RecA filament (18), Goodman and coworkers (20) published an article in which they claimed that Pol V-catalysed TLS occurs only when RecA nucleoprotein filaments assemble on separate ssDNA molecules in *trans*. A major difference between the two sets of experiments is the nature of the DNA primer/template substrate used. In their work, Goodman and coworkers (20) used a short hairpin DNA with a 3-nt-long template overhang, whereas we used a long single-stranded circular template (18). Goodman and coworkers (16) recently published another Pol V-activation model stating that the active form of Pol V is a protein complex between $UmuD'_2C$ with a single RecA monomer. Most importantly, they report that the transfer of the single RecA monomer can occur from the 3′-tip of any RecA filament, be it located in *cis* or in *trans*. Nevertheless, we wanted to assess the respective effects of providing the ssDNA-RecA filament in *cis* and/or in *trans* using a physiologically relevant single-stranded circular template similar to the probes used to measure Pol V-mediated TLS reactions in vivo. Single- and multiple-hit experiments were implemented

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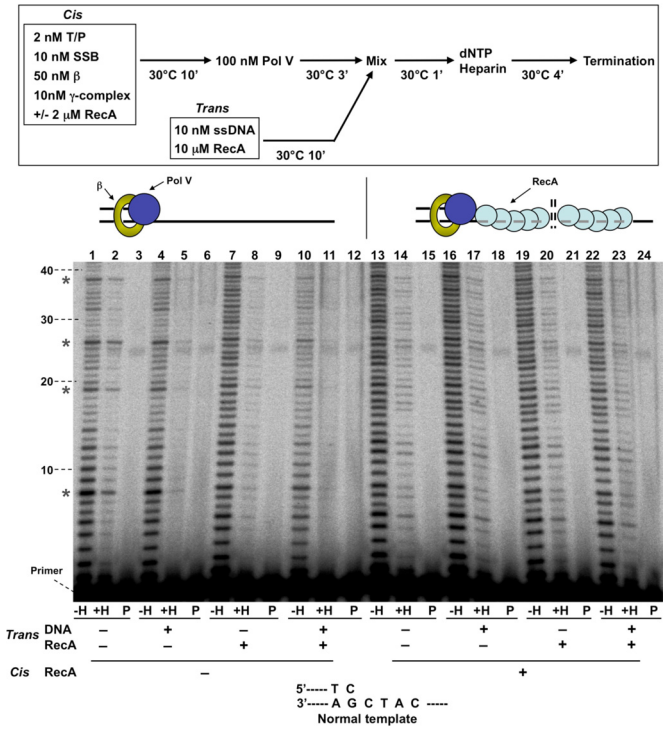

Fig. 1. Pol V requires *cis*- not *trans*-RecA filaments for stable initiation complex formation. The experimental flow chart is outlined on the top. The substrate is a lesion-free single-stranded circular plasmid DNA (sspUC-3G0 ≈2.7 kb) primed with a 5′-end ^{32}P-labeled oligonucleotide (3G context). The β-clamp is stably loaded by the γ-complex. The activity of Pol V is assessed under various RecA conditions. *Trans*-RecA is provided as ss-circular plasmid DNA (≈2.7 kb) with no primer and no SSB. Single-turnover conditions are achieved by using heparin as a trap for unbound Pol V. The efficiency of the trap is assessed by the absence of DNA synthesis when heparin is added before addition of the dNTPs (lanes P). Under single-turnover conditions (lanes +H), it can be seen that the presence of *cis*-RecA strongly stimulates Pol V activity compared with *trans*-RecA conditions that appear to inhibit the activity of Pol V. Synthesis resulting from multiple-hit events occurring during a 4-min incubation time can be seen when no heparin is added (lanes −H). The major pause sites that RecA contributes to suppress are highlighted by an asterisk.

as follows. First, we preincubated native Pol V with a primed single-stranded circular plasmid loaded with the β-clamp in the absence of dNTPs to allow Pol V to form initiation complexes. To achieve single-hit conditions, we used heparin as a trap. Indeed, heparin quenches nonloaded Pol V molecules, as well as Pol V molecules, when they dissociate from their substrate. Simultaneous addition of dNTPs and heparin, to the preformed Pol V initiation complexes, recapitulates single-hit conditions. However, multiple-hit conditions are achieved by addition of dNTPs in the absence of heparin. Under single-hit conditions, with the β-clamp alone (i.e., without RecA), Pol V synthesizes DNA fragments up to 40 nt in length (Fig. 1, lane 2). The sequence context used in this experiment ("3G" context) produces a specific band pattern presenting several strong pause sites under both single- and multiple-hit conditions (Fig. 1, lanes 1 and 2). When RecA is added to the primer-template to form a *cis*-RecA filament, total activity of Pol V is increased, and the band pattern becomes smoother as if RecA contributed to eliminate the pause sites (compare lanes 1 and 2 with 13 and 14 in Fig. 1). Addition of *trans*-RecA filaments to the reaction containing *cis*-RecA does significantly not affect the activity of Pol V (compare lanes 22 with 13, or 23 with 14 in Fig. 1). We conclude that the presence of RecA in *cis* stimulates the activity of Pol V, and contributes to overcome replication pause sites. Activation of Pol V by *cis*-RecA is thus immune to further addition of *trans*-RecA.

In contrast, in the absence of *cis*-RecA, a condition that appears to be physiologically less relevant, addition of *trans*-RecA severely inhibits the activity of Pol V probably by means of sequestering Pol V away from the initiation complexes (compare lanes 10 with 1, or 11 with 2 in Fig. 1). Together, it seems likely that the RecA filament that forms downstream from the lesion, i.e., in *cis*, is the essential cofactor that allows formation of a stable Pol V initiation complex.

The β-Clamp and the *cis*-RecA Filament Endow Pol V with the Properties Required for TLS in Vivo. We wanted to investigate the ability of *cis*-RecA filaments to endow Pol V with the capacity to read through difficult-to-replicate DNA sequences or DNA lesions under circumstances that mimic the in vivo situation as closely as possible, i.e., under single-hit conditions. In this respect, to achieve TLS it is critical for a given DNA polymerase to deliver, in a single binding event, a TLS intermediate that will withstand degradation by proofreading exonucleases. The minimal size of the TLS patch required to resist degradation by proofreading exonucleases is ≈4–5 nt (17).

We used substrates containing the *Nar*I sequence ("Nar" context) without DNA lesions or specific UV-induced TT lesions, which are physiological substrates for Pol V-mediated TLS in vivo. The Nar context is a difficult-to-replicate sequence even in the absence of lesions and a strong frameshift mutation hot spot in the presence of N-2-acetylaminofluorene (AAF) lesions (15). In the Nar context, under single-hit conditions, efficient DNA synthesis is only seen in the presence of both *cis*-RecA and the β-clamp. Kinetics of primer elongation by Pol V can be visualized as a function of time at 0.5, 1, and 2 min (+β, +RecA; Fig. 2). At 2 min and longer time points, the band pattern remains stable in sizes and intensities, illustrating the maximal activity that Pol V can produce in a single binding event. In the absence of either *cis*-RecA, or the β-clamp or both, Pol V exhibits poor activity and limited processivity. Similar conclusions can be derived from experiments conducted in the 3G sequence context (Fig. S1). Likewise, the bypass of a single UV-induced cyclobutane dimer (TT-CPD) or a TT (6-4) photoproduct is achieved under single-hit conditions when both the β-clamp and a *cis*-RecA filament are present (Fig. 3).

Quantification of the products generated by Pol V allows critical parameters such as the processivity and the overall efficiency of the reaction to be determined (Table 1). The average processivity is inferred from the plateau value reached by the "average fragment length" as defined in Table 1. On undamaged template, the average processivity of Pol V, in the presence of the β-clamp and a *cis*-RecA filament, is reached at time point 2 min and is equal to ≈25 nt (Table 1). Pol V appears to be an extremely slow DNA polymerase among all DNA polymerases of *Escherichia coli*, synthesizing on average 1 nt every 3 s. For the common DNA lesions under investigation here, the average processivity ranges from 15 to 18 nt, a value that is well above the threshold of 5 nt that prevents degradation by proofreading and insures successful lesion bypass. This slight decrease in processivity compared with undamaged template reflects the delay imposed by the bypass of the lesion (see delay estimations in Table 1).

The fraction of primer that is elongated by Pol V in a single binding event mirrors the extent of productive initiation complexes formed by Pol V. Whereas primer utilization ranges from 3 to 4% for undamaged template, it drops to ≈1% for the lesion-containing templates. All experiments with lesions have been performed with a primer ending 1 nt before the lesion (standing start). The drop in primer utilization when a lesion is present may reflect the difficulty for Pol V to properly engage into a complex with the tip of the RecA filament as a conse-

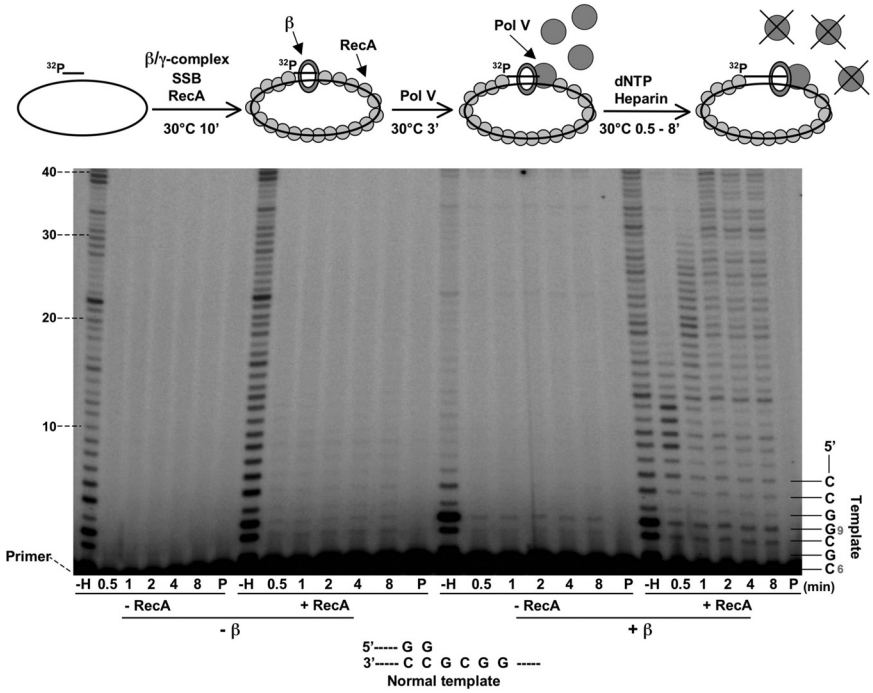

Fig. 2. In the Nar context, robust synthesis is achieved under single-turnover conditions provided that Pol V is loaded in the presence of both the β-clamp and *cis*-RecA. No other condition (i.e., no RecA and/or no β-clamp) leads to significant Pol V activity under single-hit conditions; thus, underscoring the absolute necessity of the dual interaction of Pol V with the β-clamp and RecA. The substrate is a lesion-free single-stranded circular plasmid DNA (sspUC-Nar0 ≈2.7 kb) primed with a 5′-end ^{32}P-labeled oligonucleotide (Nar context). Primer elongation activity of Pol V can be visualized as a function of time, reaching an equilibrium status at ≈2 to 4 min. In the absence of heparin (lanes −H) the total activity of Pol V is monitored under multiple hit conditions. The strong pause site at position G9 seen in the absence of RecA (+β condition) is essentially suppressed on addition of *cis*-RecA highlighting the ability of *cis*-RecA to iron out sequence difficulties.

quence of the presence of a distorting lesion in the template strand.

In conclusion, both the β-clamp and a *cis*-RecA filament are required to confer robust activity to Pol V under single-hit conditions. Only under these conditions will Pol V be able to successfully bypass a DNA lesion or a difficult-to-replicate sequence.

Pol V Initiation Complex: The β-Clamp Provides Stability While the *cis*-RecA Filament Ensures Proper Positioning. Under single-hit conditions, neither the *cis*-RecA filament nor the β-clamp alone is able to activate Pol V to a significant extent (Fig. 2; Fig. S1). To investigate the respective contributions of the β-clamp and the *cis*-RecA filament, we examined the products made under multiple-hit conditions that allow the cumulative products synthesized by Pol V during an 8-min period to be analyzed (Fig. 2; lanes −H in Fig. S1). In both Nar and 3G sequence contexts, addition of RecA permits natural pause sites to be suppressed, as observed either in the absence or presence of β-clamp. For example, in the presence of β-clamp, the strong pause site at position G9 in the Nar context is suppressed in the presence of *cis*-RecA (Fig. 2). Using a different primer, the same pause at position G9 can similarly be overcome by the addition of RecA in *cis* (Fig. S2). Although in the absence of RecA, the β-clamp has little effect on the activity of Pol V (Fig. 2) (17), its stimulation is best appreciated when RecA is present (Fig. S1). We suggest that the β-clamp per se increases the stability of the Pol V initiation complex. However, this gain in stability turns into a genuine increase in activity only in the presence of *cis*-RecA filaments. As illustrated in Fig. 4, we envision two ways a *cis*-RecA filament can increase the activity of Pol V. First, by stretching the template DNA, and second, by triggering proper positioning of Pol V with respect to its substrate via a direct interaction with the 3′-tip of the RecA filament as suggested in vivo by Devoret and coworkers (21–23), and shown in vitro (24). Indeed, genetic experiments pointed to a critical role of RecA in Pol V-mediated mutagenesis, referred to as the direct (or third) role of RecA in SOS mutagenesis (7, 10, 25). The interaction between RecA and the umuDC gene product were mapped genetically by the isolation of *recA* alleles that resist UmuDC-mediated inhibition of recombination *recA*(UmuR) (23). These mutant *recA* alleles result from amino acid substitutions that are located on the surface of the RecA protein close to the head-tail interface between RecA monomers. Subsequently, *umu* mutant alleles that restore recombination inhibition have been isolated, further illustrating the interplay between Pol V and the RecA filament (26). To maintain a permanent contact between an advancing Pol V molecule and the tip of the RecA filament, we suggest that the RecA filament "slides back" in a 3′->5′ direction, RecA monomer dissociating from the 5′-end of the filament in an ATP-catalyzed reaction (17).

In conclusion, to be functional in lesion bypass, Pol V requires the combined actions of its two cofactors. The β-clamp provides stability to the enzyme-substrate complex while the *cis*-RecA filament ensures proper positioning of the polymerase with respect to its DNA substrate.

Conclusion

In the present article, we present a compelling biochemical model that describes the way Pol V may function during TLS in vivo. On encountering of a noncoding template lesion, the replicative polymerase dissociates and the nascent 3′-end of the primer becomes a substrate for DNA polymerase specialized in TLS. Under cellular conditions, when all other DNA polymerases are present, the main challenge for a TLS polymerase such as Pol V is to synthesize, in a single binding event, a TLS patch that is long enough to "hide" the lesion and make it invisible to the proofreading activity associated with other DNA

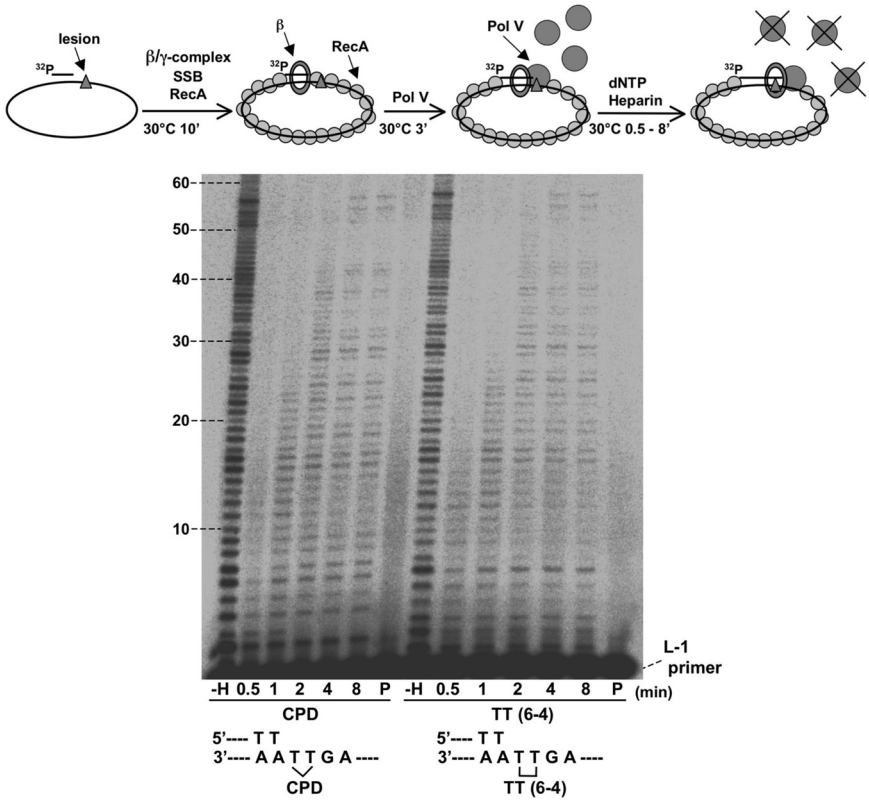

Fig. 3. Bypass of a single UV-induced cyclobutane dimer (TT-CPD) and a TT (6-4) photoproducts under single-hit conditions. The substrate is a single-stranded circular plasmid primed with an oligonucleotide with its 3′-end located 1 nt before the damaged TT site (L-1). Experimental conditions are as in Fig. 2. In the presence of both the β-clamp and *cis*-RecA filament, Pol V is processive enough to achieve lesion bypass in a single binding event. Quantitative lesion bypass parameters are indicated in Table 1. One can notice that the major pause sites are at the 5′ and 3′-T of the dimers for the CPD and the (6-4) lesions, respectively.

polymerases. We determined that a minimal patch size of 4–5 nt is enough to prevent degradation by the exonuclease associated with Pol III and Pol II and to allow extension for various lesions (17). To achieve the required processivity, Pol V needs two cofactors, the β-clamp and a RecA filament in *cis* (Fig. 5). The β-clamp, by interacting with a consensus peptide present at the

Table 1. Single-hit parameters of Pol V in the presence of the β-clamp and *cis*-RecA

Sample	Time, min	Average fragment length, nt	Average velocity, nt/s	Max. fragment length, nt	Primer utilization, %	Delay, s
Nar0	0.5	8–9	≈0.28	18	—	—
	1	17–18	≈0.29	31	—	—
	2	≈25	—	55	3.6	—
	4	≈25	—	>100†	—	—
	8	≈25	—	>100†	—	—
Nar3 (AAF)	0.5	2–3	≈0.083	13	—	—
	1	10–11	≈0.18	26	—	—
	2	≈18	—	48	1.2	24
	4	≈18	—	>61‡	—	—
	8	≈18	—	>61‡	—	—
CPD	0.5	2	≈0.067	16	—	—
	1	5–6	≈0.092	28	—	—
	2	≈15	—	44	0.79	34
	4	≈15	—	57	—	—
	8	≈15	—	57	—	—
TT (6-4)	0.5	2	≈0.067	19	—	—
	1	7–8	≈0.13	33	—	—
	2	≈15	—	48	0.68	34
	4	≈15	—	57	—	—
	8	≈15	—	57	—	—

The "average fragment length" synthesized by Pol V, at a given time, is established by summing the intensities of all bands and determining the position of the band that corresponds to 50% of the total amount synthesized. For a given template, the average fragment length increases with time and reaches a plateau value that represents the average processivity. The average velocity (nt/s) is calculated by dividing the average fragment length by time. Primer utilization represents the fraction of primer that has been elongated by Pol V during a single binding event. It reflects the proportion of productive initiation complexes formed by Pol V.

†Bands larger than 61 nt represent ≈9% of total products.

‡Bands larger than 61 nt represent ≈1% of total products. AAF data are from ref. 15.

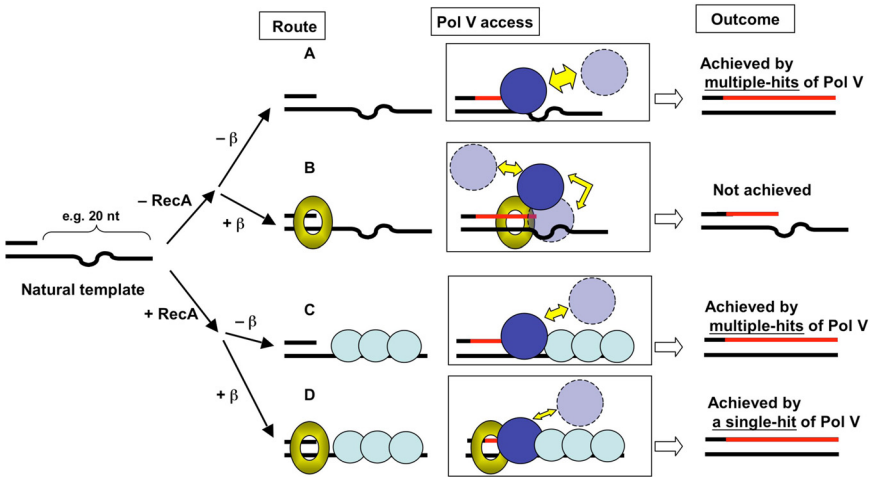

Fig. 4. The β-clamp and the *cis*-RecA filament help Pol V to read through difficult-to-replicate DNA sequence or DNA lesions (wavy line). Bypass of such a sequence is considered to be successful if the patch synthesized by Pol V in a single binding event exceeds 5 nt making it immune to exonucleolytic degradation (see main text and ref. 17). Only route D (+RecA, +β-clamp) allows Pol V achieve this goal. Together, the two cofactors endow Pol V with the properties required for lesion bypass in vivo. Pol V may also achieve read-through alone (route A) or in the presence of RecA (route C) under multiple-hit conditions. However, intermediates, displaying TLS patches shorter than 5 nt, will be formed and degraded; thus, aborting the whole bypass process. The β-clamp alone (route B) while increasing the residency time of Pol V does not appear to stimulate the bypass capacity in the absence of RecA. We suggest that on binding to the β-clamp Pol V adopts a so-called ''off'' position as described for Pol IV (31). The interaction of Pol V with the 3′-tip of the RecA filament may be essential to properly position Pol V and bring it into position ''on.'' These conditions are necessary to achieve TLS in vivo.

C-terminal end of UmuC (11, 27), increases the stability of the complex that Pol V forms with its substrate. The RecA filament that forms on the single-stranded template downstream from the lesion (*cis*-RecA) is also a key factor for Pol V activation. Our data show that it suppresses pause sites probably as a result of the stretched conformation of DNA within a RecA nucleofilament (28); thus, leveling the structural polymorphism naturally present in ssDNA. This view is supported in vivo and in vitro by data showing that RecA inhibits −2 frameshift mutations in the *Nar*I hot spot by preventing the formation of slipped intermediates (29, 30). These data strongly support the notion that a *cis*-RecA filament is indeed formed in the vicinity of a lesion. Also, we suggest that the interaction of Pol V with the tip of the RecA filament ensures proper positioning of the polymerase with respect to the template possibly by inducing an off to on switch as suggested for Pol IV (31).

The present model recapitulates in biochemical terms the essential features previously uncovered by numerous genetic studies of induced-mutagenesis in *E. coli*, namely the third role of RecA (7), as well as the critical role of the β-clamp for TLS (11). The scenario of Pol V-mediated TLS can be viewed as follows: When an exposed ssDNA region is formed downstream

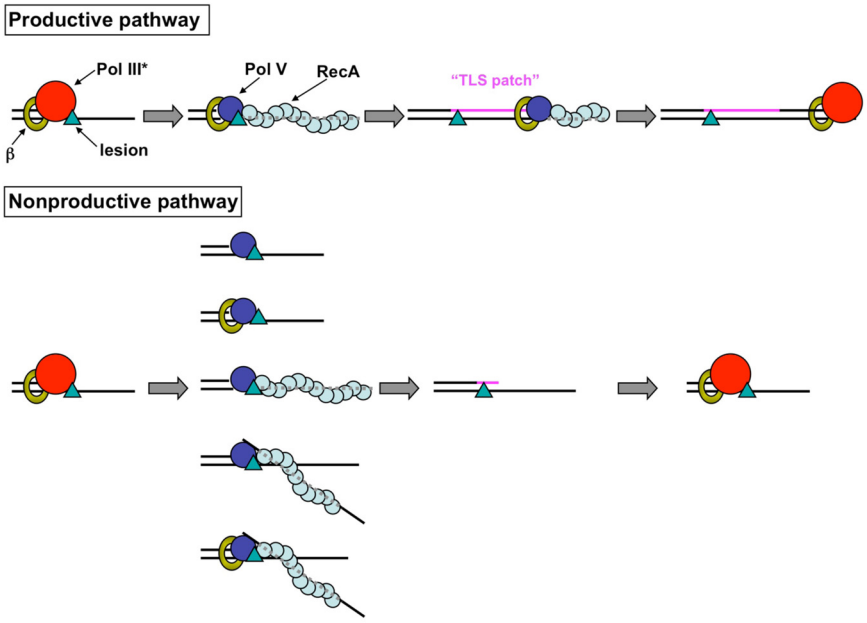

Fig. 5. A simplified model for productive Pol V-mediated TLS. Pol V needs to interact with the β-clamp and the 3′-tip of a *cis*-RecA filament to gain enough stability to ensure synthesis of a TLS patch that can subsequently be elongated by Pol III. These requirements are both necessary and sufficient. Neither the β-clamp nor a *cis*-RecA filament alone suffice. RecA filament in *trans* alone or with the β-clamp is also insufficient.

from a replication blocking lesion, it is immediately coated by SSB to ensure replication fork integrity. Subsequently, RecFOR proteins allow the exchange from SSB to RecA. The *cis*-RecA filament thus formed stimulates UmuD autocleavage into UmuD′; thus, allowing Pol V assembly during the late-phase of SOS induction. Reconstitution of TLS in vitro under conditions including stoichiometric amounts of SSB and RecA was found to completely depend on the presence of RecFOR (13). Another experimental observation points to *cis*-RecA as the critical factor for Pol V activation. When performing Pol V-mediated bypass assays, we noticed that the presence of Pol III had a net inhibitory effect on the efficiency of the TLS reaction. We suggest that repeated (and unproductive) binding events of Pol III to the blocked 3′-end of the primer disrupts the *cis*-RecA filament; thus, inhibiting the bypass activity of Pol V. Inhibition of TLS by Pol III can be relieved by addition of RecFOR and SSB proteins owing to the capacity of these proteins to rapidly reconstruct a RecA filament (13). This observation suggests that indeed RecA in *cis* is involved in Pol V activation.

Last, it is now commonly accepted that gaps are generated in the daughter strand opposite lesions in the template, and that their repair occurs behind the replication fork either by TLS or by RecF mediated homologous recombination. It should be stressed that the two pathways require the same reaction intermediate, i.e., a single-stranded DNA covered with RecA. The efficiency of the TLS reaction mediated by Pol V exhibits very poor efficiency (1% primer utilization; Table 1). The average length of the TLS patch, i.e., the processivity of the enzyme, matters more than the efficiency of the reaction per se. This concept is in striking contrast with the common view that a "good" TLS polymerase necessarily bypasses a given lesion efficiently. The low efficiency of Pol V may in fact be an additional way to limit TLS and its mutagenic consequences in letting most gaps to be filled-in by error-free homologous recombination. The present article presents an integrated view of TLS under physiologically relevant conditions in *E. coli* that may represent a paradigm for lesion bypass in other organisms.

Materials and Methods

Pol III*, Pol V, SSB, and the β-clamp were prepared as described (17, 18). The γ-complex was kindly provided from C. S. McHenry. RecA was purchased from GE healthcare. Constructions of 2.7-kb long ss-circular template DNA (lesion-free normal templates and TT photodimer containing templates) were prepared as described previously (18, 32). All primers (25-mer) were labeled by ^{32}P at the 5′-ends and purified by PAGE. ATP and dNTPs were purchased from GE healthcare. DNA replication assays were described previously (17, 18) and in the figure legends. Briefly, processivity of Pol V was measured as follows. The 2.7-kb ss-circular DNAs containing a single photodimer or without lesion (normal template) were used as templates. Templates were annealed with a 25-mer ^{32}P labeled primer, preincubated with 10 nM SSB (as a tetramer) and 2 μM RecA in the standard reaction mixture (20 mM Tris·HCl, pH 7.5/4% glycerol/8 mM DTT/80 μg/mL BSA/2.5 mM ATP/8 mM $MgCl_2$,) without dNTP for 10 min at 30 °C. When indicated, 50 nM β (as a dimer) and 10 nM γ-complex were added to the reaction. Subsequently, Pol V (100 nM) was added to form an initiation complex. After 3 min of incubation, reactions were initiated by mixing 0.1 mM dNTPs and 0.2 mg/mL heparin. Reactions were terminated at the indicated times (0.5–8 min) by adding two volumes of formamide containing 25 mM EDTA and bromophenol blue. To assess the efficiency of the heparin trap, dNTP+heparin was added to the preincubation mixture before Pol V (8 min of incubation, lanes P) or reaction mixtures were incubated without heparin (8 min of incubation, lanes −H). The products were heat denatured, separated on a 10% denaturing PAGE, and visualized on a Molecular Imager (Bio-Rad) or on a FLA-5000 (Fujifilm).

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