ARTICLES

RecA acts in *trans* to allow replication of damaged DNA by DNA polymerase V

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The DNA polymerase V (pol V) and RecA proteins are essential components of a mutagenic translesion synthesis pathway in *Escherichia coli* designed to cope with DNA damage. Previously, it has been assumed that RecA binds to the DNA template strand being copied. Here we show, however, that pol-V-catalysed translesion synthesis, in the presence or absence of the β-processivity-clamp, occurs only when RecA nucleoprotein filaments assemble or RecA protomers bind on separate single-stranded (ss)DNA molecules in *trans*. A 3′-proximal RecA filament end on *trans* DNA is essential for stimulation; however, synthesis is strengthened by further pol V–RecA interactions occurring elsewhere along a *trans* nucleoprotein filament. We suggest that *trans*-stimulation of pol V by RecA bound to ssDNA reflects a distinctive regulatory mechanism of mutation that resolves the paradox of RecA filaments assembled in *cis* on a damaged template strand obstructing translesion DNA synthesis despite the absolute requirement of RecA for SOS mutagenesis.

In 1974, Miroslav Radman proposed that *E. coli* possessed an inducible response to protect cells from the deleterious consequences of DNA damage[1]. The hypothesis was elaborated on shortly thereafter by Evelyn Witkin[2], and the "SOS response" (as it has come to be known) has been well characterized in the ensuing three decades and is now recognized to involve the upregulation of over 40 proteins engaged in DNA repair, replication and recombination under the control of the LexA repressor[3]. It has tacitly been assumed that uncoupling of leading and lagging strand replication at blocking lesions results in unwound regions of ssDNA ahead of the stalled replication fork, which serve as a platform for RecA nucleoprotein filament formation[4]. If SOS-induced non-mutagenic repair processes are insufficient to reactivate cellular DNA replication, a mutagenic phase of SOS is initiated, facilitated largely by the translesion polymerase, pol V (also known as the $UmuD'_2C$ complex)[4]. Translesion DNA synthesis (TLS) is generally successful in restarting DNA replication, and is accompanied by a large (~100-fold) increase in mutations targeted principally at sites of DNA damage[3].

Both *in vitro*[5–8] and *in vivo*[9–11], pol V activity is almost entirely dependent on the RecA protein. A RecA filament formed on damaged DNA located *cis* to pol V has thus been the focus of previous studies on the molecular mechanisms of pol-V-dependent TLS[8,12,13]. Indeed, the role of RecA in TLS has progressed through several iterations, from simply positioning $UmuD'_2C$ at the template or primer[14,15], to a moving interaction that changes as the RecA filament is displaced by pol V[12], and, most recently, as an integral component of pol V[16] (Supplementary Fig. 1). However, one crucial factor was overlooked in all of these studies—the possibility that RecA might act in *trans*, rather than in *cis*, to stimulate pol V.

Transactivation of pol V by RecA bound to ssDNA

Linear and closed-circular primer/template DNA contains either excess primer or template strands. The use of hairpin DNA instead of primer/template DNA ensures the absence of unannealed ssDNA. Pol V alone catalyses weak primer elongation on hairpin DNA containing a 3-nucleotide-long template overhang (Fig. 1a, lane 1; Supplementary Fig. 2). RecA in the presence of ATP does not stimulate pol V (Fig. 1a, lane 2). However, the addition of a non-homologous ssDNA (*trans* 80-mer) activates RecA for robust primer extension by pol V (Fig. 1a, lane 3). These data demonstrate that strong stimulation of pol V occurs when RecA is activated by a *trans* DNA molecule—that is, one that is not copied by pol V. (Supportive control data are provided in Supplementary Fig. 3.)

We examined whether transactivation is required for pol V activity when the β-processivity-clamp is included in the reaction (Fig. 1b). To ensure efficient β-clamp loading by the γ-clamp-loading complex, a hairpin with a 12-nucleotide overhang was used, and a biotin–streptavidin barrier was attached to prevent the β-clamp from sliding off. Processivity of another SOS DNA polymerase, pol IV, is enhanced (Fig. 1b, lane 5), verifying that the clamp has been loaded properly. However, pol V is barely active in the presence of the β-clamp (Fig. 1b, lane 1). RecA inhibits pol-IV-mediated synthesis in the absence or presence of the β-clamp (Fig. 1b, lanes 6 and 7, respectively), indicating that RecA is able to interact with the hairpin DNA. Nonetheless, RecA is incapable of stimulating pol V (Fig. 1b, lane 2), unless *trans* ssDNA is also present (Fig. 1b, lane 3).

Pol V typically copies past DNA lesions—for example, abasic sites ("X" in Fig. 1c–e). However, it is only able to perform TLS when not only RecA, but also ssDNA in *trans*, is present in the reaction (Fig. 1c, lanes 3 and 5). TLS is measured as the fraction of primers extended past the lesion and is enhanced by approximately threefold from 7% to 22% in the presence of the β-clamp (Fig. 1c, lanes 3 and 5). The enhancement of lesion bypass requires the presence of streptavidin-bound DNA to prevent dissociation of the β-clamp (Fig. 1c, lane 6). These data indicate that transactivation by RecA is necessary for both normal and translesion synthesis in the presence or absence of the β-clamp.

It has been a mechanistic question whether or not *bona fide* RecA filaments were required for pol V TLS[8,13] or whether smaller RecA–DNA complexes could suffice[16,17]. We extended the template overhang to 50 nucleotides to determine whether transactivation is still required when RecA filaments are formed in *cis* (Fig. 1d). As observed

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with the shorter overhangs, pol-V-catalysed primer elongation (Supplementary Fig. 4) and TLS (Fig. 1d) is essentially inactive except when RecA and ssDNA are provided in *trans*. We verified that hairpin DNA containing a 50-nucleotide overhang stimulates autocleavage of LexA, showing that RecA, which can act as a co-protease, is not excluded from the *cis* DNA (Supplementary Fig. 4).

DNA lesions can occur in the leading strand, modelled by the primer/template hairpin DNA in Fig. 1d. *In vivo*, however, pol V could also act on damaged lagging strand gaps. To address the possibility of differential activation requirements on gapped DNA substrate, we confirmed the requirement for transactivation on double hairpins with or without lesions (Supplementary Fig. 5).

Primed ssDNA circles have been used previously to characterize the effects of pol-V-catalysed TLS[7,8,18,19]. To ensure that our observations are not an anomaly related to the use of hairpin DNA, a 30-mer primer was annealed to a synthetic 240-mer circle in 1:1 stoichiometry. As observed for the hairpin substrates, pol V is barely active in the presence of RecA, the β-clamp or both (Fig. 1e, lanes 1–4; Supplementary Fig. 6). However, robust synthesis and TLS are observed when unprimed single-stranded circular DNA mimicking the presence of excess unannealed circular template is provided in *trans*, with processivity enhanced by the β-clamp (Fig. 1e, lanes 5 and 6; Supplementary Fig. 6). Thus, the addition of separate transactivating ssDNA molecules seems to be a stringent requirement for RecA-mediated stimulation of pol V synthesis on damaged and undamaged DNA templates (Fig. 1).

In vivo data indicate that gaps persist and disappear much later during the SOS response[20], so the majority of RecA–ssDNA complexes in cells induced for SOS could occur within gapped DNA. Therefore, in comparison with transactivating single-stranded linear DNA, hairpin DNA and single-stranded circular DNA, we determined that RecA bound to ssDNA gaps also transactivates pol V

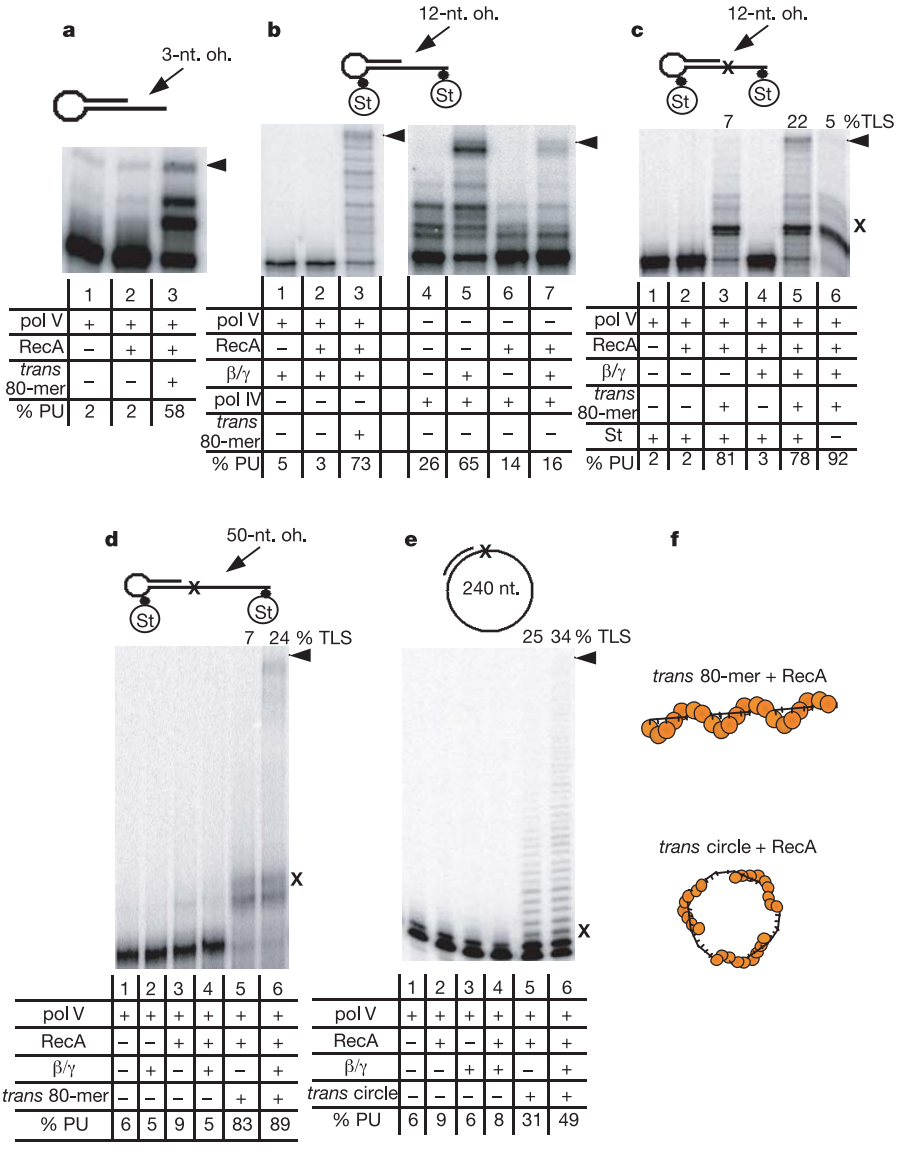
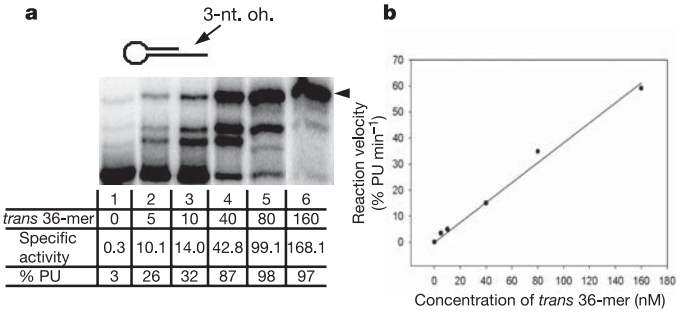


Figure 1 | RecA–ssDNA transactivation of pol V on damaged and undamaged DNA templates. a, Transactivation of pol-V-catalysed synthesis on a hairpin DNA with a 3-nucleotide (nt.)-long template overhang (oh.) by a ssDNA (80-mer)–RecA complex present in *trans* (lane 3). **b**, RecA–DNA transactivation of pol V in the presence of the β/γ-sliding-clamp and clamp-loading complex (left gel); control reactions with pol IV (5 nM) (right gel). St denotes the presence of streptavidin–biotin blocks. **c**, Transactivation of pol-V-catalysed TLS. X denotes the site of the DNA lesion (an abasic site). **d**, Transactivation of pol V synthesis on a DNA hairpin containing a 50-nucleotide-long template overhang with a lesion, X. **e**, Transactivation of pol V on damaged (X) circular DNA by RecA bound to *trans* circular DNA. **f**, Sketch of transactivating DNA. In **a**–**e**, the arrowheads indicate the position of the full-length product; % PU is the percentage of hairpin templates extended (primer utilization); transactivating DNA molecules are at 160 nM, when present; and RecA is at 4 μM, when present. In **c**–**e**, % TLS is the percentage of primers extended past the lesion, X.



	1	2	3	4	5	6
trans 36-mer	0	5	10	40	80	160
Specific activity	0.3	10.1	14.0	42.8	99.1	168.1
% PU	3	26	32	87	98	97

Figure 2 | Kinetics of pol V transactivation by RecA–ssDNA. a, Pol-V-catalysed primer utilization when copying a hairpin containing a 3-nucleotide-long template overhang was measured in the presence of ATP-γS and RecA (2 μM) at varying *trans* ssDNA (36-mer) concentrations (in nM). The specific activity of pol V is measured in pmol DNA per μg pol V per min. The arrowhead indicates the position of the full-length product. **b**, Primer extension velocity depends on *trans* DNA concentration. The kinetic data used to calculate the reaction velocity is shown in Supplementary Fig. 9.

(Supplementary Fig. 7). The degree of transactivation is greater for longer ssDNA regions. These data show that *trans*-stimulation of pol V occurs when RecA binds to linear ssDNA (Fig. 1a–f), or any form of gapped DNA (Supplementary Fig. 7).

Transactivation kinetics of pol V by RecA bound to ssDNA

To facilitate the characterization of the transactivation kinetics, reactions were performed using the slowly hydrolysable ATP-γS (Fig. 2), which inhibits filament disassembly. Synthesis was measured on 3-nucleotide-overhang hairpin DNA substrates to eliminate inhibition by the *cis* RecA filaments that can assemble only on longer ssDNA overhangs (Supplementary Fig. 8). A linear relationship is observed between the velocity of pol V synthesis and the concentration of *trans*-DNA–RecA complex. The second-order dependence of pol V activity on the *trans*-DNA–RecA complex is evidence of a bimolecular transactivation reaction (Fig. 2b). The specific activity of pol V increases 30-fold in the presence of 5 nM *trans* ssDNA, which is substoichiometric to the hairpin DNA (Fig. 2a, lane 2), compared with pol V in the absence of *trans* DNA (Fig. 2a, lane 1). A fourfold excess of *trans* ssDNA (80 nM) over hairpin DNA (20 nM) results in a 400-fold increase in the specific activity of pol V (Fig. 2a, lane 5). An eightfold excess of *trans* (160 nM) over hairpin (20 nM) DNA results in complete primer utilization within 20 min (Fig. 2, lane 6), and even within 5 min (Supplementary Fig. 9).

Pol V transactivation requires a free 3′ RecA filament end

The 3′-proximal end of the RecA nucleoprotein filament on *trans* ssDNA has a crucial stimulatory role (Fig. 3). A single-stranded 30-mer was biotinylated at the 5′ end and attached to a streptavidin-coated magnetic bead (Supplementary Fig. 10). The *trans*-activator bead strongly stimulates pol V activity (Fig. 3a, lane 2). When the biotin tag is switched to the 3′ end of the DNA, RecA will nucleate on the DNA–bead complexes with the same 5′-to-3′ directionality. In this case, however, the bead blocks access to the 3′-proximal end. This inverted filament is substantially less effective in stimulating pol V (Fig. 3a, lane 3), even though comparable amounts of RecA are bound to each of the DNA–bead complexes (Supplementary Fig. 11).

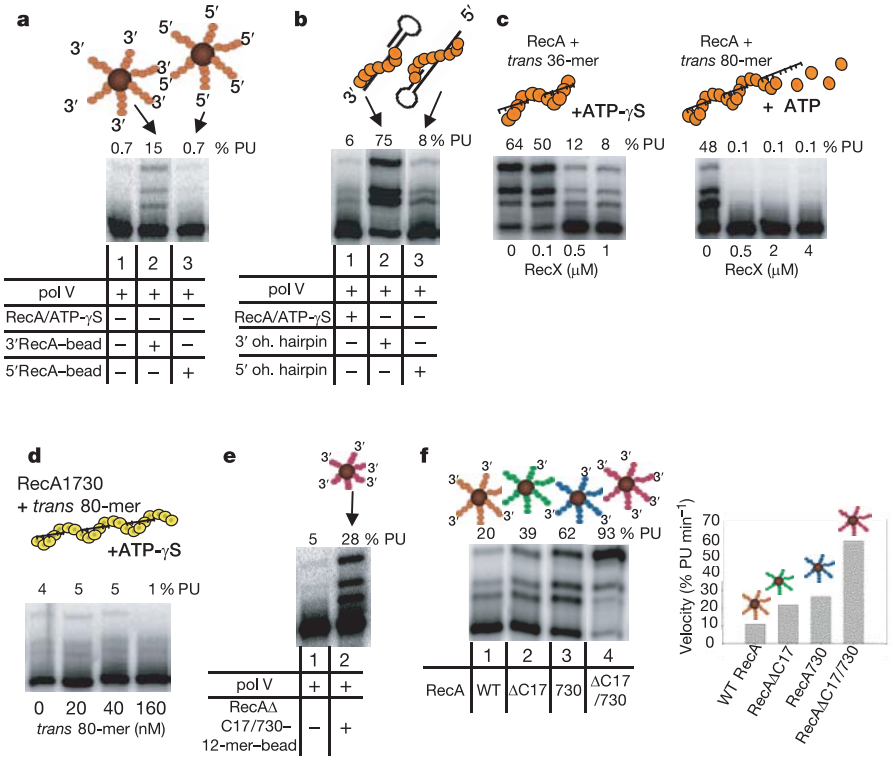


Figure 3 | A RecA filament end oriented 3′ on *trans* DNA is required for efficient stimulation of pol V. a, RecA protein (~4 μM) immobilized on a DNA–bead complex (~1 μM of DNA molecules bound to the bead) with exposed 3′-proximal RecA ends causes strong transactivation of pol V. A filament with exposed 5′ ends shows weak transactivation. **b**, RecA (2 μM) bound to a hairpin DNA containing a 3′ overhang stimulates pol V much more than one with a 5′ overhang. **c**, RecX protein inhibits pol V synthesis in the presence of RecA (1 μM), *trans* 36-mer (80 nM) and ATP-γS (left panel), or RecA (4 μM), *trans* 80-mer (160 nM) and ATP (right panel). **d**, Mutant RecA1730 (4 μM) in the presence of ATP-γS and *trans* 80-mer (160 nM) is deficient in transactivation. **e**, Mutant RecAΔC17/730(E38K) (~1 μM) immobilized on a 12-mer-DNA–bead complex (~1 μM of DNA molecules bound to the bead) causes robust transactivation. **f**, Wild-type (WT) and mutant RecA proteins (~4 μM) immobilized on a 30-nucleotide-DNA–bead complex stimulate pol V synthesis with significantly different efficiencies. Hairpin primer/template DNA contains a 3-nucleotide-long template overhang (see diagram in Fig. 2a). (See Supplementary Methods and Supplementary Fig. 10 for preparation of RecA–DNA bead complexes.)

The requirement for an accessible 3′-proximal RecA filament end on ssDNA for pol V stimulation was confirmed by using hairpin oligonucleotides with 3′ or 5′ overhangs as the *trans*-activator DNA (Fig. 3b). Presumably, RecA filamentation on hairpins with a 5′ overhang continues into the double-stranded regions, therefore excluding a 3′ end on the ssDNA. The regulatory protein[21] RecX blocks RecA filament extension by binding 3′ to filament ends[22]. RecX also inactivates RecA transactivation of pol V, most likely via a steric block, even when RecA is formed on the DNA with the slowly hydrolysable ATP-γS before the addition of RecX (Fig. 3c, left panel). RecX is even more inhibitory in the presence of ATP (Fig. 3c, right panel). The RecA1730 mutant protein confers an SOS non-mutable phenotype[10,23], and is unable to stimulate pol V activity *in vitro*[16]. The same holds true for the transactivation reactions (Fig. 3d). In the RecA1730 mutant, a phenylalanine replaces a serine at amino-acid residue 117, a position that is located at the surface facing 3′ on a RecA filament end[15].

Hierarchy of pol V transactivation by RecA mutants

Mutant RecAΔC17/730(E38K)[24] associates tightly with ssDNA[16] and is able to transactivate pol V while attached to a 12-mer-DNA–bead complex (Fig. 3e, lane 2). RecA–12-mer-DNA complexes do not provide sufficient space for a complete helical turn of a RecA filament. Notably, owing to the presence of the beads, these short complexes cannot stack to form longer filaments. Thus, the data support the idea that no more than a small RecA protomer is necessary for transactivation. Nevertheless, a longer *trans* ssDNA oligomer (30 nucleotides) increases RecA stimulatory activity (Fig. 3f, left panel, lane 4).

The three mutant RecA proteins RecAΔC17[24], RecA730(E38K)[25] and RecAΔC17/730(E38K)[16] all bind tighter to DNA than wild-type RecA, and for that reason were believed to enhance RecA function. However, RecA binding properties are irrelevant in experiments with magnetic beads because comparable amounts of RecA protein are stably bound to the DNA–bead complex (Supplementary Fig. 11). The various RecA proteins show a hierarchy in pol V stimulation (Fig. 3f). Mutant RecAΔC17 is missing its carboxy-terminal lobe, which otherwise occupies parts of the helical groove in a RecA filament[26]. Therefore, this deletion may facilitate access to the groove for proteins such as UmuD′, as has been modelled previously[27]. RecAΔC17 doubles the reaction velocity (Fig. 3f, right panel) and primer utilization by pol V (Fig. 3f, lane 2) compared with wild-type RecA. It is therefore tempting to speculate that the additional stimulation seen in the presence of RecAΔC17 is attributable to a facilitated interaction between UmuD′ and the helical groove of a RecA filament, as suggested in a previous study using electron microscopy[27].

Discussion

Here we propose a model for the role of pol V in SOS DNA-damage-induced mutagenesis in which an interaction between pol V and the surface of a RecA protomer or 3′-proximal RecA filament end is both necessary and sufficient for lesion bypass (Fig. 4a, orange–red circle). On the basis of *in vivo* studies with RecA mutants, Devoret and co-workers[15,28,29] proposed that a 3′-proximal RecA filament end interacting with UmuD′C (later identified as pol V ($UmuD'_2C$)[4]) at a site of DNA damage in *cis* was necessary for TLS. Conversely, the central conclusion of the transactivation model is that interactions between pol V and RecA responsible for synthesis stimulation must occur while the proteins are bound to different molecules of DNA. This effect was obscured in previous studies investigating pol-V-catalysed TLS from different groups[7,8,13,18,19,30], including ours[5,6,12,16,17], where some fraction of the ssDNA could always have bound RecA capable of activating pol V complexes on other primer/template molecules in *trans*.

The activity of pol V increases linearly with the concentration of ssDNA supplied in *trans* (Fig. 2b). The bimolecular nature of the reaction represents strong kinetic evidence that stimulation of pol V by RecA *per se* is dependent on a second *trans* DNA molecule bound to RecA. Complete hairpin DNA extension is observed on DNA substrates that preclude RecA inhibition in *cis* (Fig. 2a). The presence or absence of the β-clamp does not alter the requirement that RecA bound to DNA in *trans* is required for pol-V-catalysed TLS (Fig. 1).

RecA nucleation on ssDNA can occur at any segment, and is followed by filament extension in the 3′ direction[31] while disassembly, driven by ATP hydrolysis, occurs with the same directionality, thus creating new nucleation sites. Therefore, free 3′-proximal RecA filament ends will continuously be generated on ssDNA, such as (for example) circular DNA (Fig. 1e) or gapped DNA (Supplementary Fig. 7), and can serve as transactivation docks for pol V. Presumably, the longer the single-stranded region stretches, the more RecA filaments are able to initiate, thereby increasing the number of available 3′-proximal ends. Notably, a gapped circle containing longer ssDNA (Supplementary Fig. 6) or an unprimed single-stranded 240-mer circle (Fig. 1e, lane 5) provided in *trans* increases the extent of pol V stimulation.

An unambiguous consequence of a stalling replisome or replication fork collapse on an encounter with a DNA lesion is the creation of ssDNA regions. This is a key event for the initiation of the SOS response. Accurate repair mechanisms, such as recombinational repair or excision repair, all of which involve or create ssDNA, try to cope with the damage before inducing mutagenic pol V. However, gaps persist[20] and disappear only very late during the SOS response. Coincidentally, pol V is also produced late in the SOS response, presumably as a last resort to facilitate cell survival.

We suggest that regulation of SOS mutagenesis requires elevated levels of ssDNA in *trans* for optimal pol V activation (Fig. 2). Simple access to a stalled replication fork and high amounts of RecA are insufficient for appreciable pol-V-catalysed synthesis. Mutagenesis is restricted to times of extensive DNA damage: for example, when numerous ssDNA gaps are present, thus increasing the chances for a ssDNA gap transactivator (Fig. 4b) to stimulate pol V on a separate replication fork. Any segment on ssDNA, if sufficiently long, can serve as a RecA filament platform (Fig. 4b). Once the damage is bypassed and extended, ssDNA disappears and pol V activities decline. The requirement for RecA during pol-V-catalysed TLS *in*

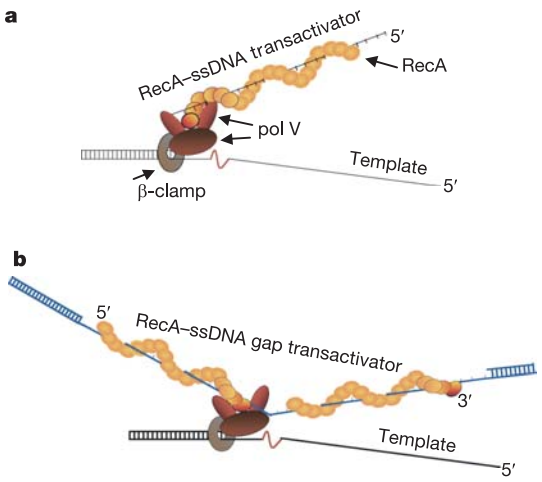


Figure 4 | Models depicting RecA–DNA transactivation of pol-V-catalysed translesion DNA synthesis. a, RecA–ssDNA (orange circles) is required for pol V ($UmuD'_2C$) to copy undamaged and damaged DNA templates. The essential feature of the transactivation model is that a RecA–DNA complex is formed on ssDNA that is not being copied by pol V. If RecA were to bind instead to the template strand, *cis* to pol V, then TLS would be blocked. **b**, A variation on the model shows how transactivation of pol V might occur by a RecA filament formed in gapped DNA (blue). A damaged DNA base is shown as a distortion in the template strand (brown squiggle).

vivo and *in vitro* seemed incompatible with the observation that RecA filaments formed in *cis* on the template being copied can block DNA synthesis[12,16] (Supplementary Fig. 12). Activation of pol V by RecA–ssDNA in *trans* resolves this incompatibility and seems to constitute a new type of polymerase regulatory mechanism.

METHODS

Nucleotide incorporation on DNA primer templates. The polymerase activity was examined by extending primer/template hairpin DNA ^{32}P-labelled at the 5′ end, or ^{32}P-labelled primed circular DNA, as indicated. Substrate DNA was preincubated with β-processivity-factor/γ-clamp-loading complex (when present) and pol V before addition of RecA, *trans* DNA and dNTPs. Preincubation of *trans* DNA with RecA is redundant, as RecA will be attracted to DNA that is longer and/or in molar excess over primer/template DNA. RecA concentrations, *trans* DNA length or concentrations, and the presence of either ATP or ATP-γS are indicated in the figures and figure legends. The sequences of template and transactivating DNAs are provided in Supplementary Fig. 13. For DNA beads, a biotinylated oligomer was attached to a streptavidin-coated magnetic bead (Bangs Laboratories) following the manufacturer's protocol. RecA was immobilized on the beads with ATP-γS as described in Supplementary Fig. 10, and added to the reaction at a concentration of ~1 μM in total DNA molecules. Unless indicated otherwise, reactions were carried out for 20 min at 37 °C and the synthesis products were separated on a 20% denaturing polyacrylamide gel. The gel band intensities were measured by phosphorimaging with IMAGEQUANT software (Molecular Dynamics) and primer utilization was computed from the integrated gel band intensities of extended hairpin DNA or primer DNA. TLS was calculated by dividing the number of hairpins extended past the abasic site by the total number of extended hairpins. Transactivation kinetics curves (Supplementary Fig. 9) were analysed using Sigma Plot. For further details on the methods used in this study, see Supplementary Methods.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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