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Mutations for Worse or Better: Low-Fidelity DNA Synthesis by SOS DNA Polymerase V Is a Tightly Regulated Double-Edged Sword

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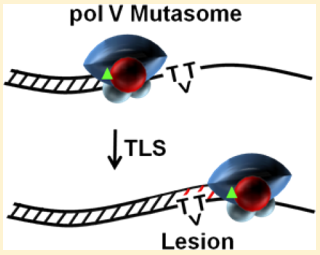
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ABSTRACT: 1953, the year of Watson and Crick, bore witness to a less acclaimed yet highly influential discovery. Jean Weigle demonstrated that upon infection of *Escherichia coli*, λ phage deactivated by UV radiation, and thus unable to form progeny, could be reactivated by irradiation of the bacterial host. Evelyn Witkin and Miroslav Radman later revealed the presence of the SOS regulon. The more than 40 regulon genes are repressed by LexA protein and induced by the coproteolytic cleavage of LexA, catalyzed by RecA protein bound to single-stranded DNA, the RecA* nucleoprotein filament. Several SOS-induced proteins are engaged in repairing both cellular and extracellular damaged DNA. There's no "free lunch", however, because error-free repair is accompanied by error-prone translesion DNA synthesis (TLS), involving *E. coli* DNA polymerase V ($UmuD'_2C$) and RecA*. This review describes the biochemical mechanisms of pol V-mediated TLS. pol V is active only as a mutasomal complex, pol V Mut = $UmuD'_2C$-RecA-ATP. RecA* donates a single RecA subunit to pol V. We highlight three recent insights. (1) pol V Mut has an intrinsic DNA-dependent ATPase activity that governs polymerase binding and dissociation from DNA. (2) Active and inactive states of pol V Mut are determined at least in part by the distinct interactions between RecA and UmuC. (3) pol V is activated by RecA*, not at a blocked replisome, but at the inner cell membrane.



Mutations for worse are responsible for death in microorganisms and disease in humans. Mutations for better are a central driving force for fitness and evolution in all forms of life. *Escherichia coli* DNA polymerase V (pol V) initiates the lion's share of mutations targeted at DNA template lesions, including those caused by UV radiation and a variety of chemicals that can damage DNA.[1−4] The initial description of the polymerase function of the *E. coli* DinB (pol IV) and UmuDC (pol V) proteins in 1999[5−7] was part of the broader discovery of a new and widespread family of specialized DNA polymerases.[8] These are the Y family DNA polymerases, which promote a process called translesion DNA synthesis (TLS). These polymerases exhibit a relaxed specificity, increasing the level of mutagenesis when they function. In *E. coli*, pol V is responsible for virtually all of the mutagenesis that accompanies the SOS response.[1−4,9,10]

The *umuD* and *umuC* genes encoding error-prone pol V ($UmuD'_2C$) are induced in response to DNA damage as part of the SOS regulon.[1,2] The promoter for the *umuDC* operon possesses a near perfect match with the consensus LexA repressor binding motif, resulting in its relatively late SOS induction. The operon is not fully derepressed until ∼15 min after DNA damage,[11] and the $UmuD'_2C$ proteins themselves do not accumulate until ∼45 min after damage, because of the rapid proteolysis of the Umu proteins.[12] Once formed, pol V is largely resistant to proteolysis, and the enzyme copies past DNA template lesions that normally arrest replication by the high-fidelity pol III.[13] Low-fidelity pol V lacks intrinsic 3′−5′ exonucleolytic proofreading, so it cannot correct any mistakes it makes during TLS. However, if the pol V-dependent TLS tract is limited, then any misincorporated nucleotides introduced by pol V during TLS may be subject to extrinsic proofreading by the *dnaQ*-encoded ε subunit of pol III. The extent of extrinsic proofreading may be temporarily suppressed because of an increase in the size of nucleotide pools that accompany the SOS response[14] and may allow pol III to elongate pol V-catalyzed misincorporations and thereby help sustain them as damage-induced mutations in the *E. coli* genome.[14]

RecA bound to single-stranded DNA (ssDNA) in the presence of ATP as an active and extended nucleoprotein filament is often termed RecA*.[15] RecA has multiple roles in the SOS response, and its effects are paramount to both SOS regulation and downstream implementation. RecA* appears when RecA binds to the ssDNA byproduct of DNA damage

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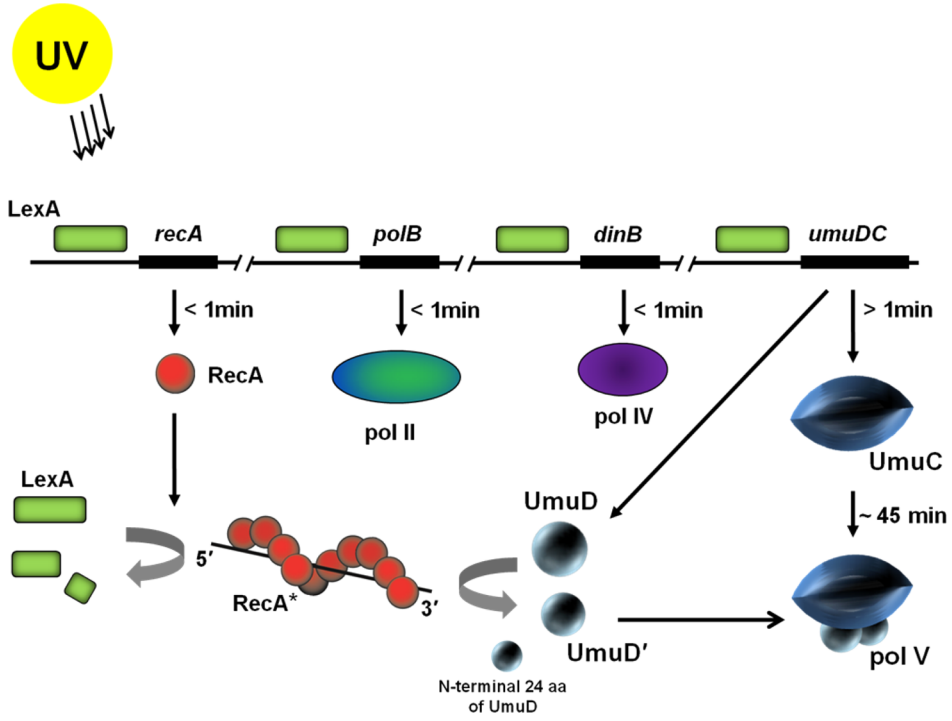


Figure 1. Induction of TLS DNA polymerases during the SOS response in *E. coli*. The LexA repressor (green box) binds to a 20 bp consensus sequence in the operator region of the SOS genes, suppressing their expression. Upon UV irradiation, expression of RecA (red spheres) is induced almost immediately after DNA damage occurs. RecA forms a nucleoprotein filament on ssDNA (RecA*) and functions as a coprotease to cleave and thereby inactivate the LexA repressor. The binding affinities of LexA for operon sequences of various SOS genes determine their induction time. Proteins with operators bound weakly by LexA, such as RecA, pol II, and pol IV, are induced soon after DNA damage occurs, whereas proteins with strongly bound operators, such as the *umuDC* operon, are induced later (>1 min).[11] The umuDC operon is fully derepressed ~15 min after DNA damage;[11] however, because of rapid proteolysis of the Umu proteins,[12] the UmuD′$_2$C complexes themselves do not accumulate until ~45 min after damage. RecA* facilitates cleavage of UmuD (15 kDa) to UmuD′ (12 kDa), removing the first 24 amino acids from the N-terminus of UmuD. Two molecules of UmuD′ (UmuD′$_2$) form a physical complex with UmuC (48 kDa) forming pol V (72 kDa).

events. Acting as a coprotease, RecA* induces the SOS response by facilitating the autocatalytic cleavage of the LexA repressor protein to upregulate transcription of the SOS regulon genes (Figure 1).[16] The RecA* coprotease activity is again needed to form pol V by facilitating the autocatalytic cleavage of the UmuD protein to a shorter and mutagenically active UmuD′[9,17,18] that dimerizes and binds to UmuC[19] (Figure 1). Finally, UmuD′$_2$C is activated by transfer of a RecA subunit from RecA* to UmuD′$_2$C to form pol V Mut (UmuD′$_2$C-RecA-ATP), the active TLS polymerase.[20]

Until 1998, UmuD′$_2$C was widely considered an auxiliary factor that, with RecA, somehow modulated DNA polymerase III to effect mutagenic DNA synthesis across lesions in the DNA template. However, when incubated in the presence of RecA*, the same UmuD′$_2$C promoted TLS in the absence of any added DNA polymerase.[13] Most notably, pol III holoenzyme (HE) composed of pol III core ($\alpha\varepsilon\theta$ subunits), β sliding clamp, and γ clamp loader complex was absent.[13] Soon thereafter, UmuD′$_2$C was identified as pol V;[5] its catalytic activity resides in its UmuC subunit.[5,6] The "auxillary factor" therefore evolved into a bona fide DNA polymerase;[8] many more have since been identified in prokaryotes and eukaryotes.[8,21,22]

Despite its demonstrable polymerase activity, pol V was largely catalytically inactive in the absence of RecA*.[5,13] This review is centered on why and how RecA* is needed for pol V activity. Distinct from its role as a coprotease in converting UmuD to UmuD′, RecA* plays a unique and (for RecA) uncharacteristically direct role in pol V function. RecA*, acting as a catalyst, transfers a RecA monomer from its 3′-proximal filament tip along with a molecule of ATP to inactive pol V to form an activated pol V mutasome, pol V Mut (a UmuD′$_2$C-RecA-ATP complex).[20] ATP may be transferred in concert with RecA or might perhaps bind separately.

The biochemical roles of RecA and ATP in regulating pol V Mut activity have come into focus since 2009, with surprises at every step. There have been limited studies demonstrating the role of RecA outside nucleoprotein filaments, such as its ability to bind to DinB and modulate polymerase fidelity.[23,24] However, pol V Mut is the only instance in which a physical complex with a RecA monomer is absolutely essential for DNA polymerase activity.[20] Recent advances add to the dynamic. pol V Mut has an intrinsic DNA-dependent ATPase activity that appears to act as an internal clock to regulate polymerase processivity.[25] The RecA* activation of pol V is spatially regulated, most likely occurring at the cell membrane.[26] The last observation connects current research with the mid-1980s observation of Evelyn Witkin that RecA* is localized on the membrane in *E. coli* lysates.[27] More recently, it has become clear that initiation of the SOS response depends upon that same RecA membrane localization.[28] The robust field of SOS "error-prone" repair initiated by Witkin[29] and Miroslav Radman[30] just keeps on giving, thus providing impetus to reflect on the earlier genetically based TLS models in light of recent biochemical and live cell imaging discoveries.

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TLS BEFORE 1998

The Bridges–Woodgate two-step lesion bypass model[31] involving the UmuDC proteins and RecA (Figure 2A) provides a mechanistic framework for tracing the progress in dissecting the biochemical basis of pol V-catalyzed TLS. In the first step, nucleotide misincorporation at the site of DNA damage, pol III copies the lesion, requiring the action of RecA protein. In the second step, lesion bypass, UmuC and UmuD proteins are needed to facilitate continued DNA synthesis on the priming end opposite the lesion.[31] The importance of the *umuDC* operon-encoded damage-induced genes for SOS mutagenesis[1,2,32] was established before the Bridges–Woodgate model was proposed in 1985. Cells having defects in either *umuC* or *umuD* are UV nonmutable.[1] A direct role for RecA in pol III-catalyzed misincorporation was assumed in the model on the basis of *recA* mutant strains that exhibited differences in their RecA* coprotease activity; those with good coprotease activity promoted higher levels of mutagenesis, while those with poor coprotease activity promoted low levels of mutagenesis.[33] Three years later, the discovery that UmuD′ was the active form of UmuD, formed by the coproteolytic activity of RecA*, provided an alternative explanation for the role of RecA in SOS mutagenesis.[17,18] The stage was set for another wrinkle.

Devoret and colleagues[34] soon demonstrated that RecA* had a separate and direct role in TLS. The RecA S117F mutant, named RecA1730, was able to catalyze homologous recombination, use its coproteolytic function to cleave the LexA repressor to turn on SOS, and cleave UmuD to form UmuD′. However, SOS mutagenesis did not occur. This newly discovered direct role for RecA in TLS led to a new mutasome model proposed by Echols and Goodman[35] (Figure 2B). Relying on the known filament forming properties of RecA, the model placed a RecA nucleoprotein filament in *cis*, at the lesion site on the template strand being copied. It was assumed that this filament would somehow facilitate replication past the lesion with reduced fidelity. This might occur by inhibiting pol III proofreading[36] or perhaps by reducing insertion specificity by stretching the ssDNA at the stalled replication fork (Figure 2B). The mutagenically active UmuD′ was included in this "Echols mutasome" model.[35] Thus, although many parts of the model proved to be incorrect, all of the main actors in the story were finally present. Quoting from our contemporary 1990 review,[35] "What is the direct role of RecA in mutagenesis? At this point we can make reasonable guesses, but the answer awaits a biochemical reconstitution of translesion replication".

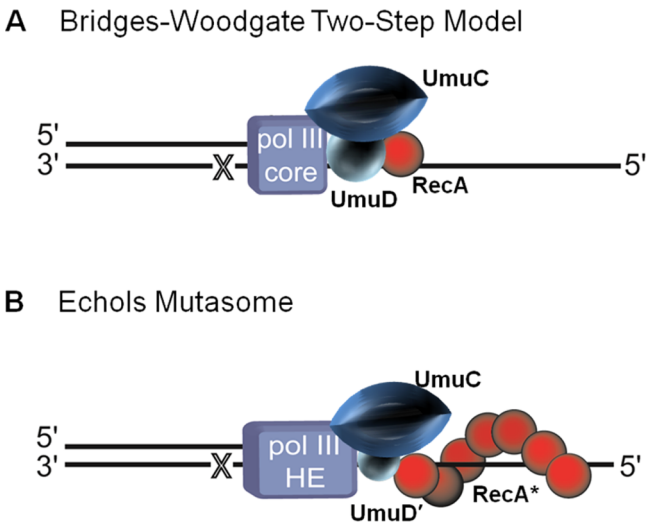


Figure 2. Translesion synthesis (TLS) models before 1998. (A) Bridges–Woodgate two-step model.[31] In this model, translesion synthesis (TLS) is catalyzed by DNA polymerase (pol) III that requires a RecA molecule for nucleotide insertion opposite a template lesion (X) and then requires the UV mutagenesis gene products UmuDC to copy past the lesion. (B) Echols mutasome model.[35] The RecA nucleoprotein filament (RecA*) assembles in *cis* at a lesion site (X) on the template strand of DNA being copied. A multiprotein complex, including pol III holoenzyme, UmuC, and UmuD′, is then recruited to the DNA, allowing TLS resulting in mutations targeted opposite the lesion.

BIOCHEMICAL RECONSTITUTION OF TLS

In 1989, minuscule amounts of soluble UmuC were obtained by refolding the purified and denatured protein.[19] Soon after that, detectable levels of TLS were generated in a reconstituted system *in vitro* with UmuC, UmuD′, pol III, and RecA.[37] However, it was not until 1998 when a soluble UmuD′$_2$C complex was isolated[13] and the biochemical reconstitution of TLS with purified SOS proteins achieved.[13,38] With the exception of UmuC, the individual components in the sketch of the mutasome model (Figure 2B), RecA*, UmuD′, and pol III HE were available as purified aqueous soluble proteins. Native UmuC, on the other hand, was insoluble in aqueous solution.[19] Overexpression of UmuC and UmuD′ in the absence of chromosomal UmuC and most importantly UmuD allowed the purification of UmuC and UmuD′ as a soluble heterotrimeric complex, UmuD′$_2$C.[39] Parenthetically, although it was not appreciated at the time, it was essential to delete the chromosomal *umuD* gene to prevent formation of a stable and interfering UmuD′D complex that triggers the release and precipitation of UmuC.[40] Consistent with the biochemically recalcitrant nature of UmuC, we now know that UmuC resides on the cell membrane and only enters the cytosol upon binding to UmuD′$_2$.[26]

Each of the required SOS mutagenesis protein components identified genetically was now in hand in a highly purified soluble form: UmuD′$_2$C and RecA*, presumably all acting to somehow alter the activity of pol III HE. These proteins were used to copy primer-template (p/t) DNA containing a site-directed template (abasic) lesion.[13,38] Robust synthesis on the damaged DNA template ensued. Unexpectedly, TLS occurred in the absence of the pol III core.[13] Indeed, pol III acted as a competitive inhibitor to UmuD′$_2$C at the TLS step,[13] and that was because UmuD′$_2$C is a DNA polymerase.[5,6] Examination of this TLS activity opened new questions about the role of RecA. The pol V-catalyzed DNA synthesis was diminished markedly in the absence of RecA*.[5,13] What was RecA* doing, and where was it doing it?

TLS 1998–2006: RECA* *CIS* ACTIVATION

The elegantly simple two-step model proposed in 1985 (Figure 2A) provided a focus for research for more than 20 years. It was flexible enough to incorporate each new biochemical advance in the period. In the "mutasome" model,[35] a molecule of RecA was replaced by a RecA* nucleoprotein filament, which was assumed to assemble at a DNA template lesion downstream of a stalled replication fork (Figure 2B). The subsequent identification of UmuD′$_2$C as a TLS polymerase resulted in a model in which pol III blocked at a lesion (Figure 3, left panel)

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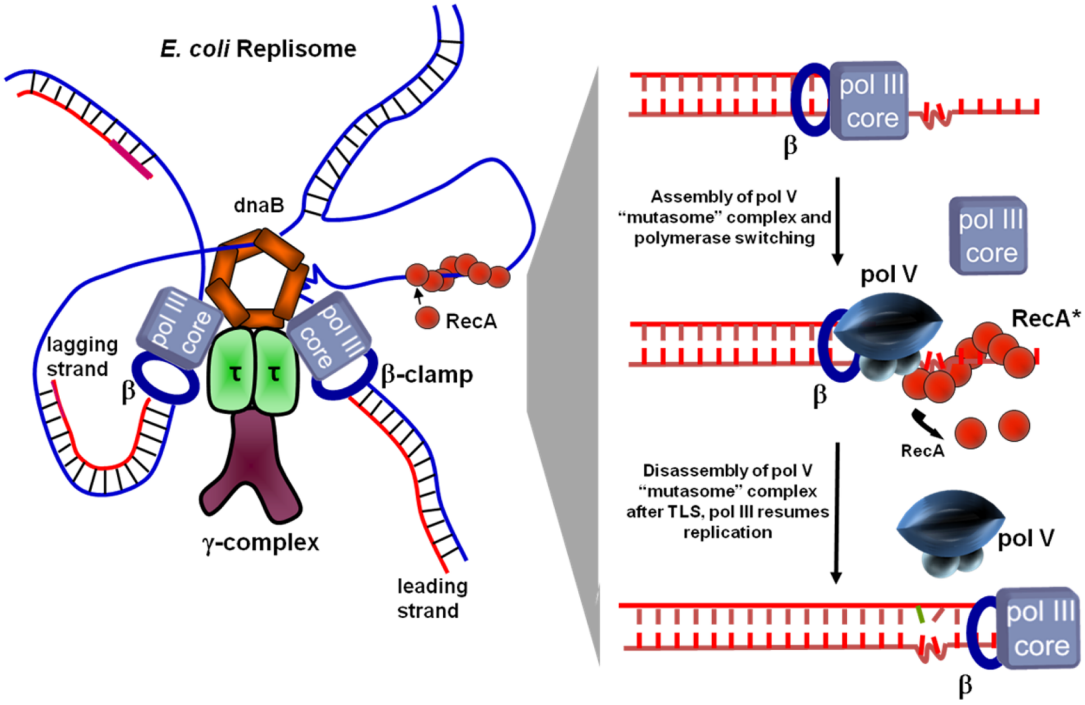


Figure 3. TLS *cis* activation model. A model of an *E. coli* replisome stalled at a lesion site is depicted in the left panel, and individual TLS steps are shown in the right panel. DNA pol III stalls at the lesion site, and DnaB helicase unwinds dsDNA ahead of the blocked fork, resulting in regions of ssDNA where RecA* assembles in *cis*. The *cis* activation model requires polymerase switching. DNA pol V replaces pol III core on the β clamp and bypasses lesions with concurrent 3′ → 5′ displacement of RecA* formed ahead of the lesion by pol V. Following TLS, pol III core replaces pol V to continue rapid replication of undamaged DNA downstream of the lesion.

was replaced on the β-clamp by pol V (Figure 3, right panel). Although modified to include newly discovered biochemical components, the common thread of each ensuing model remained the location of RecA* on the template strand immediately downstream of, and perhaps even encompassing, the lesion (Figure 3). This has been defined as the *cis* activation model for TLS, because RecA* is presumed to act in *cis* on the strand being copied to shepherd a stalled polymerase past the lesion.[41−43]

Figure 3 illustrates individual steps in TLS via *cis* activation by RecA*. Initially, an advancing replication fork containing pol III HE becomes stalled at a lesion. Leading- and lagging-strand replication become uncoupled. Continued unwinding of dsDNA ahead of the blocked fork by the DnaB helicase results in a region of ssDNA downstream of the lesion on which RecA* can assemble (Figure 3, left panel). pol V replaces pol III core on the β clamp and performs TLS accompanied presumably by progressive and concurrent 3′ → 5′ disassembly of RecA*.[41] Following TLS, pol III core would replace pol V to resume rapid DNA synthesis on undamaged DNA downstream from the lesion (Figure 3, right panel).

Given the formation and requirement for RecA* nucleoprotein filaments, *cis* activation models seemed intuitively obvious to many investigators, including us. If RecA* is required for TLS, its location should be in the single-strand DNA gap at the lesion, where it can interact with pol V. However, this construct had multiple problems. While one could visualize how the presence of RecA* might stimulate lesion bypass by interacting with pol III core, e.g., perhaps by inhibiting ε exonuclease proofreading, there was no obvious biochemical rationale for the workings of RecA* in conjunction with pol V. A RecA* filament on the DNA is a potentially formidable barrier to the progress of any DNA polymerase. The model envisioned displacement of RecA subunits at the RecA filament end where filament growth normally predominates.[44−50] Active RecA filament displacement from this filament end was proposed as a part of a model, where pol V was dubbed a "cowcatcher".[41] The DNA synthesis observed seemed to require such a displacement but was not directly demonstrated as an activity of pol V. The subsequent demonstration of *trans* activation of pol V[51] provided an explanation of the pol V-mediated DNA synthesis results without invoking RecA displacement in *cis*. Currently, direct RecA displacement function has been demonstrated only for specialized helicases such as UvrD[52] and PcrA.[53,54]

TLS AFTER 2006: RECA* *TRANS* ACTIVATION

The *cis* activation construct began to give way in 2006.[51] When pol V was used to copy p/t DNA in the form of a hairpin, containing a very short three-nucleotide template overhang (Figure 4), replication of the short template by pol V required the presence of RecA* assembled on a separate ssDNA molecule acting in *trans* (Figure 4). The new *trans* protocol also

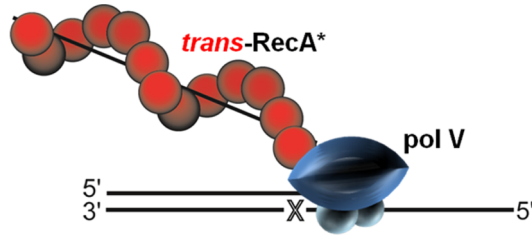


Figure 4. DNA pol V transactivation by RecA*. RecA* is assembled in *trans* on ssDNA separate from p/t DNA. DNA pol V is then activated by *trans*-RecA*, resulting in TLS.

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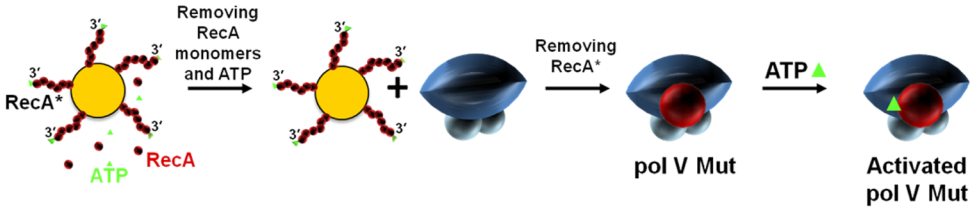


Figure 5. pol V mutasome (Mut). pol V Mut isolation is a multistep procedure requiring direct contact between pol V and RecA*. First, RecA* is formed on ssDNA covalently attached to agarose beads, and unbound RecA monomers (red spheres) and ATP (green triangle) are removed by extensive washing. pol V is then added. Interaction of pol V with RecA* leads to transfer of a RecA subunit from the 3′-proximal tip of RecA* to form pol V Mut. Activated pol V Mut is composed of UmuC-UmuD′$_2$-RecA-ATP.

increased the overall efficiency of primer utilization dramatically and consistently. Apparently, assembling the RecA* filament in *cis* had a constrained reaction efficiency in many experiments.[55,56] The fraction of p/t DNA extended by pol V was directly proportional to the concentration of *trans* RecA* in accord with second-order kinetics.[51] The three-nucleotide template cannot support the assembly of a *cis*-RecA* filament (at least one that is restricted to the ssDNA region) because the footprint of a single RecA molecule is itself three nucleotides in length. How might a transactivated pol V Mut behave were it to encounter RecA* assembled downstream of a lesion? The presence of RecA* acts as a block to DNA synthesis and would presumably need to be displaced, which could involve SSB, as proposed in our previous "cowcatcher" model for TLS,[41] or perhaps more likely by the UvrD[52] or PcrA[53,54] helicases.

The pol V *trans* activation method offered a straightforward approach to determining the molecular basis for the enigmatic requirement for RecA* during TLS. By locating pol V and RecA* on separate macromolecules, one had only to mix the pol V together with *trans*-RecA* and ask what happens. To answer this question, it was necessary to completely separate the *trans*-RecA* from pol V following incubation, to eliminate any possibility of continued transactivation of pol V. Quantitative separation was achieved by forming RecA* filaments on ssDNA that was covalently linked to streptavidin-coated agarose beads, spinning the beads out of solution to remove the *trans*-RecA*, and isolating a putative modified form of pol V from the supernatant (Figure 5).[20]

RecA*−pol V Transactivation Mechanism. When pol V (UmuD′$_2$C) was exposed to RecA* in *trans*, a molecule of RecA accompanied by an ATP molecule was transferred from the 3′-proximal tip of the RecA nucleoprotein filament to form an active mutasomal complex, now termed pol V Mut. The final activated pol V Mut complex is UmuD′$_2$C-RecA-ATP[20] (Figure 5). The transfer of RecA to pol V was revealed using MALS (multiangle light scattering).[20] This powerful technique provides an absolute measurement of molecular mass.[57] UmuD′$_2$C (72 kDa)-RecA (38 kDa) has a predicted molecular mass of 110 kDa, in excellent agreement with the value of 113 kDa obtained with MALS. pol V in the absence of bound RecA has a molecular mass of 73 kDa, in close agreement with its predicted value. A PAGE analysis verified that RecA was present in the light scattering peak corresponding to pol V Mut, but absent in the pol V peak.

pol V Mut Copies DNA in the Absence of RecA*. The identification of active pol V Mut as a stand-alone polymerase meant that undamaged and damaged DNA could, for the first time, be replicated efficiently in the absence of RecA*. To form pol V Mut, there are stringent spatial restrictions on the RecA*−pol V interaction. The key amino acids involved in the productive transfer of RecA to pol V include RecA residues 112−117; these are located at the solvent-exposed surface near the 3′-tip of RecA*, in the proximity of UmuC (e.g., N113 forms a cross-link with UmuC).[58] A D112R mutation causes a 4-fold reduction in activity, and a D112R/N113R double mutant abolishes polymerase activity. Most importantly, from a historical perspective, pol V Mut assembled with Devoret's SOS nonmutable RecA mutant, UmuD′$_2$C-RecA(S117F),[34] is catalytically dead,[20] thus most likely accounting for the absence of damage-induced mutagenesis in RecA1730 (S117F) mutant cells *in vivo*.[34] In addition, the RecA surface represented by residues 112−117 is the site of mutations that relieve inhibition of RecA-mediated recombination by overexpressed UmuC and UmuD proteins.[59]

The RecA subunit of activated pol V Mut must come from the 3′-proximal end of the RecA* filament. Although a molecule of RecA can also be transferred from the 5′-proximal filament tip to UmuD′$_2$C, that complex lacks detectable polymerase activity.[20] A stable complex can also be formed between RecA and UmuD′$_2$C in the absence of RecA*, but this form of UmuD′$_2$C-RecA has no detectable polymerase activity in the presence or absence of ATP.[20,60] Thus, although RecA* is required to turn on the SOS regulon (Figure 1) and convert UmuD to UmuD′ to form pol V = UmuD′$_2$C, the precursor form of pol V Mut (Figure 1), the most direct role of RecA* during SOS mutagenesis resides in the formation of a functional pol V Mut by transferring a RecA subunit to pol V.

pol V Mut Requires Bound ATPγS/ATP. pol V Mut assembles in a 1:2:1:1 stoichiometry with UmuC, UmuD′, RecA, and ATP/ATPγS.[20] The transfer of one molecule of RecA from the 3′-proximal tip of RecA* to UmuD′$_2$C occurred concomitantly with the binding of one molecule of ATPγS.[20] This slowly hydrolyzing nucleotide cofactor ensures stable RecA* filament assembly, because filaments formed with ATP undergo a dynamic assembly−disassembly process resulting from ATP hydrolysis.[46,61,62] However, in the original transactivation assays, the stoichiometric binding of ATPγS to UmuD′$_2$C had actually taken place adventitiously from the supernatant containing pol V Mut (Figure 5), and not directly during the transfer of RecA.[25] The role of the nucleotide cofactor in the pol V Mut complex, if any, was not understood at that time.

A more efficient transactivation protocol was developed, utilizing cyanogen bromide Sepharose beads with RecA* bound to covalently attached ssDNA in the presence of ATPγS, along with the use of a spin column to separate pol V Mut from the *trans*-RecA*, unbound RecA, and the free ATPγS/ATP used to form RecA*.[25] We recovered a form of pol V Mut (UmuD′$_2$C-RecA) devoid of both ATPγS/ATP and polymerase activity.[25] Restoration of pol V Mut activity occurred upon addition of

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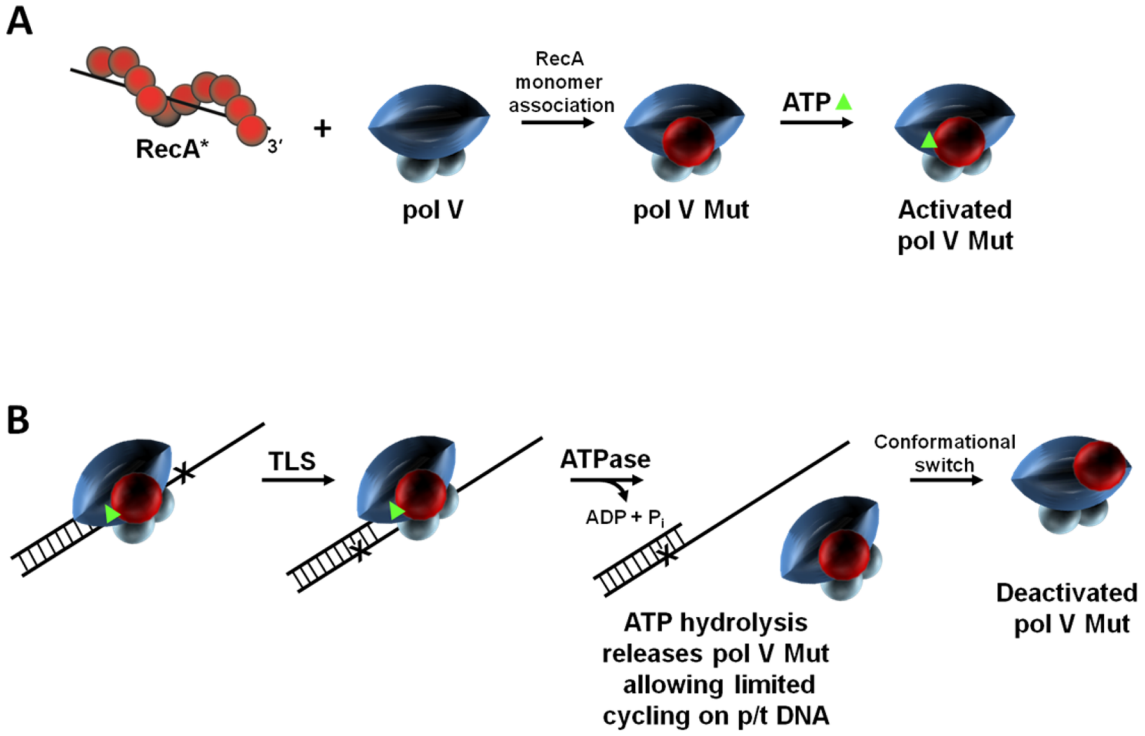


Figure 6. RecA and ATP regulation of pol V Mut activity. pol V activity is regulated in multiple steps. (A) First, a RecA subunit is transferred to pol V from the 3′-proximal tip of RecA* to form pol V Mut. pol V Mut is activated by binding a molecule of ATP (green triangle). (B) Activated pol V Mut associates with p/t DNA, and DNA synthesis proceeds until ATP is hydrolyzed. pol V Mut is a unique DNA-dependent ATPase, where a single ATP hydrolytic event leads to dissociation of the enzyme from p/t DNA, followed by limited cycling on p/t DNA. After DNA synthesis, pol V Mut becomes deactivated but can be reactivated by exposure to a new RecA*. We propose that active and deactivated pol V Mut are determined by the location of RecA on pol V.

new ATPγS/ATP. Therefore, an adenosine nucleoside triphosphate cofactor ATP, dATP, or ATPγS was necessary as an integral component of pol V Mut to catalyze DNA synthesis on undamaged and damaged (TLS) DNA templates. Other nucleotide forms are unable to substitute for ATPγS or ATP; e.g., pol V Mut is not activated by GTP, ADP, or dTTP.[25]

POL V MUT IS A DNA-DEPENDENT ATPASE

The molecular basis for the A nucleotide cofactor requirement was soon revealed. pol V Mut cannot bind to a DNA primer-3′-end and, hence, has no polymerase activity, unless a molecule of ATPγS/ATP is present in the complex.[25] More remarkably, pol V Mut has an intrinsic DNA-dependent ATPase activity.[25] This activity is distinct from the canonical DNA-dependent ATPase of RecA*. pol V Mut retains its ATPase activity when assembled using a mutant RecA* (RecAK72R), which essentially eliminates RecA*-dependent ATPase activity.[25,63,64]

Playing the reciprocal role for ATP in binding to p/t DNA, the ATPase is required to release pol V Mut from a primer 3′-end. There is a very close correspondence of ATP hydrolysis rate $[(54 \pm 9) \times 10^{-3}\ s^{-1}]$ to the first-order pol V Mut-p/t DNA release rate $[(53 \pm 2.5) \times 10^{-3}\ s^{-1}]$, strongly suggesting that the hydrolysis of one ATP molecule triggers the release of pol V Mut from p/t DNA.[25] In contrast, pol V Mut remains stably bound to DNA in the presence of ATPγS where hydrolysis does not occur.[25] The DNA-dependent ATPase can be viewed as an internal clock that limits pol V Mut processivity to minimize the occurrence of mutations on undamaged DNA following TLS. The individual steps in the ATP-ATPase regulation of pol V Mut activity are shown in Figure 6.

SPATIAL REGULATION OF POL V MUT ACTIVATION *IN VIVO*

The activity of pol V Mut is clearly regulated on many levels. Formation of the enzyme requires an elaborate activation process with multiple steps. The lifetime of the active complex on DNA is regulated by an intrinsic ATPase activity,[25] but this is not the end of the regulation story. Yet another level of regulation of pol V Mut, this one spatial, has recently been revealed in living cells by fluorescence imaging at single-molecule (sm) resolution.[26] This study addressed the cellular location of pol V Mut after induction of the SOS regulon. Replacement of wild-type *umuC* with fluorescently labeled *umuC*-mKate2 showed that UmuC is synthesized and localized at the inner cell membrane ~45 min after UV irradiation. Thereafter, UmuC remains sequestered on the membrane and is released into the cytosol only after RecA*-mediated UmuD → UmuD′ cleavage occurs. The membrane association explains why UmuC is inherently insoluble in aqueous solution and is solubilized by binding with $UmuD'_2$[39] to form pol V.[5,13] In accord with the biochemical data, live cell imaging shows that UmuC stays bound to the membrane in a noncleavable *umuD*(K97A)[9] mutant strain that cannot form UmuD′. The fluorescently labeled pol V Mut (UmuC-mKate2) is partially functional in the cell, promoting an increased level of DNA damage-induced spontaneous mutagenesis.[26] The role of the membrane in the activation mechanism of pol V, as now understood, is shown in Figure 7. Most of the current information centers on the membrane sequestration of UmuC prior to its incorporation into active complexes. RecA protein is also present in the membrane,[27,28] but it is not clear if

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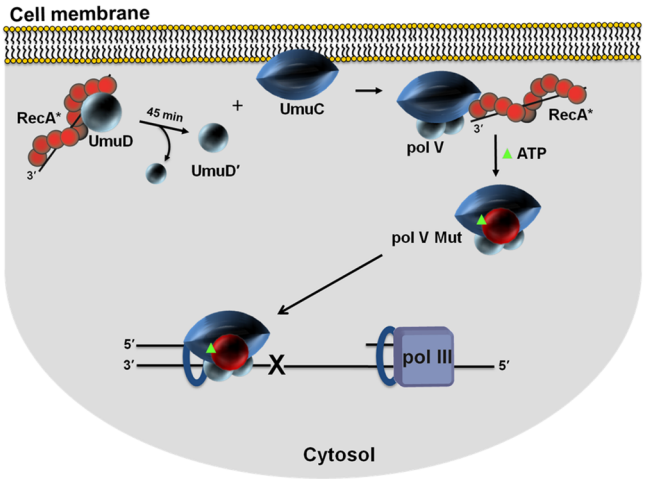


Figure 7. Spatial regulation of pol V Mut activity *in vivo*. Sketch depicting cell membrane localization of UmuC. Single-molecule live cell fluorescence imaging data indicate that after an initial delay post-UV irradiation, UmuC is synthesized and localized on the inner cell membrane. UmuC is released into the cytosol only after it interacts with $UmuD'_2$. As RecA and UmuD are found in or near the membrane, additional steps in the formation of pol V Mut ($UmuD'_2C$-RecA-ATP) may occur in the proximity of the membrane. It is not clear what functionality may be associated with the RecA protein found in the membrane. Active RecA*, as studied to date, consists of RecA protein filaments bound to DNA. Sequestration of UmuC at the membrane provides additional levels of pol V Mut regulation. A delay between the production of UmuC and the formation of cytosolic pol V Mut provides time for error-free DNA repair to occur before mutagenic pol V Mut lesion bypass is necessary.

that RecA is in a form that can activate pol V. It is possible that UmuC is removed from the membrane by association with $UmuD'_2$ and later activated to functional pol V Mut after diffusion and interaction with RecA* bound to the bacterial chromosome. It is also possible that additional steps occur at the membrane, such as UmuD cleavage and activation to pol V Mut, involving currently unknown functions of membrane-associated RecA protein.

Once active pol V Mut is formed, where does it go? Instead of a complex activated at the site of a lesion, we now see a freely diffusible activated pol V Mut complex. In principle, it could act at a stalled replication fork or in a single strand and lesion-containing gap left behind after restart of that stalled fork.[65] Following the release of pol V into the cytosol, the colocalization of pol V mutasomes with replication forks depends on the RecA variant that is present.[26] When pol V Mut contains wild-type RecA, i.e., $UmuD'_2C\text{-}RecA_{WT}$-ATP, there is no colocalization between pol V Mut and pol III HE above what would be predicted if the two species were located randomly throughout the cell (~5%).[26] The absence of colocalization between pol V Mut and pol III HE in replication foci suggests that either the polymerases form a very transient ternary complex, with pol V Mut bound for <30 ms (the imaging rate used was 30 Hz, and there were no indications of transient focus formation), or the presumed exchange of the high-fidelity pol III HE blocked at a DNA damage site with the low-fidelity pol V Mut to permit TLS may in fact not be occurring. The result calls into question the widely held view that mass action binding of pol V occurs uniquely at stalled replication forks. Instead, when pol III skips over a replication-blocking lesion and initiates DNA synthesis downstream, a β-clamp may be left at the lesion site to which pol V Mut can bind and catalyze TLS. Our *in vivo* observation comports with the pol III lesion-skipping model proposed by Yeeles and Marians[65] based on biochemical data showing that when impeded by DNA damage, pol III jumps past the damage site, leaving behind a β-clamp bound to a 3′-primer end. The polymerase then reinitiates synthesis on the same template strand downstream from the lesion on a reloaded β-clamp using an RNA primer formed by DnaG primase.[66,67]

In contrast to the behavior observed in cells with wild-type RecA, a significant proportion of pol V Mut foci (27 ± 3%) colocalize with pol III HE in cells containing the constitutively expressed RecA E38K mutant (previously termed RecA730).[27] These data suggest that pol V can replace pol III during normal DNA synthesis on undamaged templates. Constitutively expressed in undamaged cells, RecA E38K will activate pol V in an environment where lesion-containing gaps are less common. Its subsequent action at replication forks may help explain the 100-fold increase in the level of pol V Mut-catalyzed SOS mutagenesis in the absence of exogenous DNA damage observed in cells constitutively induced for RecA E38K.[10] When undamaged DNA is being copied, the fidelity of proofreading-deficient pol V Mut, deoxynucleotide misincorporation, $\sim 10^{-2}$ to 10^{-3},[4] is typically >1000-fold lower than that of proofreading-proficient pol III HE.[68]

SOS MUTAGENESIS MODEL 2015

We suggest a unified model for the regulation of SOS mutagenesis that integrates the original *in vivo* genetically based concepts to *in vitro* biochemical data obtained since 2009 with current live cell imaging microscopy that furnishes essential physiological missing links coupling the old genetics and new biochemistry. The model is divided into three distinct mechanistic stages. Stage 1 embodies numerous genetic studies showing that *umuDC* is transcribed as a late SOS function (Figure 1). Stage 2 portrays combined genetic and biochemical studies depicting the temporally retarded RecA*-mediated cleavage of UmuD → UmuD′, required for SOS mutagenesis (Figure 7), and the identification of the activated form of pol V ($UmuD'_2C$) as pol V Mut ($UmuD'_2C$-RecA-ATP) (Figure 5). The individual biochemical steps to convert pol V to activated pol V Mut are presented in Figure 6. Stage 3 illustrates how ATP and ATP hydrolysis regulate pol V Mut activity. The steps for ATP activation and ATPase deactivation of pol V Mut are shown in Figure 6. This third stage describing a unique internal regulation mechanism governing pol V Mut activity presumably limits error-prone DNA synthesis to include TLS and short regions of undamaged DNA in the vicinity of a damaged template base. The mutational load associated with pol V is thus minimized.

When it was discovered that pol V could be activated by RecA* acting in *trans* (Figure 4) and did not need to be located on ssDNA proximal to a DNA damage site being copied (see, e.g., Figure 3), the question of the cellular location of these events had no obvious answer. Things became a bit easier to explain when the molecular mechanisms of RecA* trans-activation was established (Figure 5). A stand-alone form of pol V was identified that, once formed, works in the absence RecA* (Figure 6), yet the salient question of where in the cell pol V encountered a transactivating RecA* nuclear protein filament if not activated by RecA* at a blocked replication fork as originally proposed (Figures 2 and 3) remained. At the cell membrane seems likely to be the answer on the basis of the

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recent live cell imaging studies,[26] thus providing a directly visualized missing link between the old genetics,[27] new biochemistry,[26,28] and cell physiology.

A MYOPIC LOOK BEYOND 2016

Given the many unforeseeable twists encountered in the story of this system to date, any look ahead is a bit adventurous. Akin to a toggle switch, pol V Mut turns on and off repeatedly with RecA staying bound when in activated and deactivated states (Figure 6B).[20] Activated pol V Mut (Figure 6A) can perform limited numbers of DNA synthesis cycles before it is deactivated (Figure 6B). If needed, deactivated pol V Mut can be reactivated by fresh RecA* that exchanges an "old" RecA monomer for a new one, but presumably in a different position in relation to UmuC.[58] When SOS is dialed down, a reduced availability of RecA* would presumably ensure that the deactivated form of pol V Mut is unlikely to be reactivated.

The location of RecA in relation to UmuC in activated and deactivated conformations is the next issue to resolve. The dynamics of RecA-UmuC switching could be visualized at single-molecule resolution, in real time, using TIRF-FRET microscopy, provided that a dual-labeled pol V Mut (e.g., Cy3-UmuC and Cy5-RecA) remains active. Upon a first stab at amino acid cross-linking, tandem mass spectrometry analysis found that contacts between RecA and UmuC differed significantly in active and inactive forms of pol V Mut.[58] A high-resolution crystal structure for even just one of the two pol V Muts, live or dead, would prove to be invaluable. Parenthetically, however, despite the rapid advances in cryo EM, pol V Mut (110 kDa) is currently too small to be resolved.

pol V Mut's intrinsic ATPase appears to be unique, lacking Walker A or B motifs. RecA*'s ATPase seems not germane because pol V Mut assembled with an ATPase-deficient RecA K72R mutant, pol V Mut E38K/K72R, has an avid DNA-dependent ATPase.[25] Thus, a current challenge is to identify the interacting surfaces between RecA and UmuD′$_2$C that form this potentially new type of hybrid ATPase active site. Recently, we have used desthiobiotin-ATP cross-linking[69] and tandem mass spectrometry to identify a lysine residue in UmuC (K403) that contributes to ATP binding-initiated DNA synthesis and to ATPase activity. This is admittedly a small beginning.

Apart from TLS, pol V Mut has an existential role in bacterial fitness and evolution, as do its two companion SOS-induced pols II and IV (Figure 1).[70,71] pols II and IV are present at high constitutive levels (50 and 400 molecules/cell, respectively) in the absence of external stressors, whereas pol V Mut is present at no more than ∼10 molecules per cell.[26,72] However, each contributes to approximately the same extent in ensuring cell survival by generating life-sustaining mutations, with different spectral signatures.[70,71] The cell survival data using all possible combinations of SOS polymerase genetic backgrounds suggest that pol II works most effectively during exponential growth, with pols V and IV taking over to maximize fitness in stationary and deep-stationary phases.[70,71] Live cell imaging is ideally suited to visualizing when and under what conditions (e.g., with or without UV) any of the SOS polymerases colocalize with pol III, and further to determining whether the presence or absence of each SOS polymerase alters the competition for binding to DNA. The power of the live cell imaging techniques comes into full play here because individual cells can be studied as part of an ensemble of cells. Here, as before, live cell imaging can provide a missing link among genetics, biochemistry, and cell physiology.

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ABBREVIATIONS

UV, ultraviolet light; ssDNA, single-stranded DNA; TLS, translesion synthesis; nt, nucleotide; DNA pol V, DNA polymerase V; pol V Mut, polymerase V mutasome; pol III HE, polymerase III holoenzyme; ATPγS, adenosine 5′-(3-thiotriphosphate) tetralithium salt; RecA*, RecA nucleoprotein filament; MALS, multiangle light scattering; PAGE, polyacrylamide gel electrophoresis; sm, single-molecule; TIRF, total internal reflection fluorescence; FRET, Förster resonance energy transfer.

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