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Simple and efficient purification of *Escherichia coli* DNA polymerase V: Cofactor requirements for optimal activity and processivity *in vitro*

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Abstract

Most damage induced mutagenesis in *Escherichia coli* is dependent upon the $UmuD'_2C$ protein complex, which comprises DNA polymerase V (pol V). Biochemical characterization of pol V has been hindered by the fact that the enzyme is notoriously difficult to purify, largely because overproduced UmuC is insoluble. Here, we report a simple and efficient protocol for the rapid purification of milligram quantities of pol V from just 4 L of bacterial culture. Rather than over producing the UmuC protein, it was expressed at low basal levels, while $UmuD'_2$ was expressed *in trans* from a high copy-number plasmid with an inducible promoter. We have also developed strategies to purify the β-clamp and γ-clamp loader free from contaminating polymerases. Using these highly purified proteins, we determined the cofactor requirements for optimal activity of pol V *in vitro* and found that pol V shows robust activity on an SSB-coated circular DNA template in the presence of the β/γ-complex and a RecA nucleoprotein filament (RecA*) formed *in trans*. This strong activity was attributed to the unexpectedly high processivity of pol V Mut ($UmuD'_2C \cdot$ RecA · ATP), which was efficiently recruited to a primer terminus by SSB.

Keywords

UmuC; UmuD′; SOS mutagenesis; Y-family DNA polymerase; Mutagenesis; Translesion DNA synthesis

1. Introduction

Most damage-induced mutagenesis in *Escherichia coli* is dependent on pol V, encoded by the *umuDC* operon [1,2]. Based upon genetic studies, it was originally hypothesized that the Umu proteins were replication accessory factors that enabled pol III to bypass DNA lesions in a two-step process (incorporation/extension) [3,4]. This hypothesis was initially difficult

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Conflict of interest
The authors declare that they have no conflict of interest.

to test biochemically, as the overexpressed UmuC protein forms insoluble inclusion bodies. The UmuC protein was first purified in 1989, but only after the protein had been denatured and renatured [5]. Nevertheless, the renatured UmuC protein was shown to interact with $UmuD'_2$ to form a stable $UmuD'_2C$ complex of ~70 kDa [5]. The first soluble preparation of $UmuD'_2C$ was reported in 1996 [6]. Subsequent studies revealed that rather than being an accessory factor to pol III, the $UmuD'_2C$ complex possesses intrinsic polymerase activity, and was duly termed *E. coli* pol V [7,8]. Pol V has intrinsically weak DNA polymerase activity, but its catalytic activity can be stimulated *in vitro* in the presence the β-processivity clamp, RecA protein bound to ssDNA, and single-stranded-binding (SSB) protein [9-12].

Since 1996, the protocols to purify soluble $UmuD'_2C$ (pol V) have improved [10,13], but the purification process is still complex and requires multiple steps that yields only a few milligrams of pure protein from hundreds of liters of induced starting culture. A Maltose-Binding Protein (MBP)–UmuC fusion protein has also been purified, but the ~45 kDa MBP-tag is resistant to cleavage and the recombinant MBP–UmuC fusion protein exhibits different cofactor requirements compared to $UmuD'_2C$ [8,10].

We are interested in further understanding the structural and biochemical basis for pol V-dependent translesion DNA synthesis. To do so, we would like to characterize mutant variants of *E. coli* pol V and its orthologs at the biochemical level. However, the existing pol V purification protocols would be unlikely to yield sufficient material for our studies. As a consequence, we have developed a novel strategy to purify wild-type pol V, mutant pol V variants and pol V orthologs, rapidly and efficiently in high yield and purity. Indeed, using such an approach, milligram quantities of highly purified pol V can be purified from as little as 4 L of starting *E. coli* culture. We have also developed novel strategies to purify the β-clamp and γ-clamp loader complex free from any contaminating polymerase and/or exonuclease activity. Using these reagents, we have determined the cofactor requirements and optimal reaction conditions for efficient and processive DNA synthesis by pol V on an ~3.0 kb circular, single-stranded template [14].

2. Materials and methods

2.1. E. coli expression strains

The strains and plasmids used in this study are shown in Table 1. The *E. coli* B strain, RW644, was constructed by P1 transduction [15] of the Δ(*umuDC596*)::*ermGT*, Δ *polB1*::Ωspec Δ*dinB61*::*ble* alleles from EC8 [16], DV08 [17], and AR25 [18] respectively, into BL21(λDE3) (EMD Biosciences, Madison, WI). Transduction of the various alleles was confirmed by PCR analysis [19]. DV38(λDE3) was made by lysogenization of DV38 [17] with λDE3 according to the manufacturer's instructions (EMD Biosciences, Madison, WI).

2.2. Construction of plasmids expressing high levels of soluble E. coli pol V

pHUC25 was constructed in four steps. First, a *umuC* fragment was PCR amplified from pRW134 [20] using primers NdeI-UmuC-F and XhoI-UmuC-R (Table 2), that append an NdeI site and hexahistidine tag at the 5′ end of *umuC*, as well as a downstream 3′ XhoI site. The PCR amplicon was digested with NdeI and XhoI and cloned into the NdeI–XhoI sites of pET22b (EMD Biosciences, Madison, WI), to generate pHUC23. Next, a *umuD′* fragment, including ribosome binding site (RBS) was PCR amplified from pEC48 [21] using primers umuD′-F and umuD′-Xho-R. These primers add a XhoI site at both ends of the 374 bp amplicon. The XhoI fragment was then cloned into the unique XhoI site of plasmid pHUC23 and a XbaI–PsiI fragment from plasmid pHUC23 containing the RBS for *umuC*, His-tagged

umuC, RBS for *umuD′*, *umuD′* and T7 terminator, was isolated. pSCKL1 in which the chloramphenicol acetyl transferase gene in pBB528 [22] is replaced by the aminoglycoside-3′-O-phosphotransferase gene, was generated by blunt-end ligation of AlwNI–PsiI fragment of pET28a in place of the short SfcI fragment of pBB528. Finally, the XbaI–PsiI fragment from pHUC23 was cloned into the EcoRI site of pSCKL1 by blunt-end ligation. The resulting plasmid, called pHUC25, expresses UmuC (1–422 amino acids) with an N-terminal tag of 7 amino acids (*i.e.* MHHHHHH), and UmuD′ (25–140 aa of UmuD) with an N-terminal methionine (Fig. 1). Note, however, that the modified *umu* operon does not contain a promoter and is, therefore, expressed at low basal levels.

pARAD2 was constructed by cloning the PCR-amplified *UmuD′* gene (with its RBS) from pEC48 using primers UmuD′-F and UmuD′-R, into the EcoRI site of pBAD24 [23] by blunt-end ligation. The pMB1 origin of replication was changed to a high-copy number pUC origin of replication by replacing the ClaI–BfuAI fragment of pBAD24 with the large PvuII fragment of pBluescript II KS+ by blunt-end ligation (Fig. 1).

2.3. Construction of plasmids to test the in vivo activity of His-tagged pol V

pSCD2 was constructed by replacing the NdeI–HindIII fragment of pJM227 [24] with an NdeI–EagI fragment that contains the His-UmuC and UmuD′ region of pHUC25 after filling the respective HindIII and EagI recessed ends with Klenow polymerase. pSCD1 was constructed by deleting the His-tag fragment from pSCD2 by PCR-based site-directed mutagenesis. pSCD2-D101N was constructed by swapping the SalI–HindIII fragment of pSCD2 with a corresponding region of pRW392, containing the *umuC104* (D101N) mutation [7]. pSCD2-DEAA was constructed by introducing the D101A and E102A mutations (GATGAG to GCGGCC) into *umuC* on pSCD2 by PCR-based site-directed mutagenesis.

2.4. Construction of low-copy number plasmids expressing the β clamp and γ-clamp-loader complex

Plasmid pHBETA3 was constructed by PCR amplification of the *dnaN* gene from pSJS9 [25] with primers AR127 and AR128 (Table 2). The amplicon was subsequently digested with NdeI and HindIII and cloned into the NdeI–HindIII site of pET16b. The pMB1 origin of pET16b was changed to that of pSC101, by replacing the AlwNI–PshAI fragment of pET16b with the 1877 bp SfcI–AfeI fragment from pGB2 by blunt-end ligation. pHBETA3 is therefore a low-copy number plasmid that replicates in cells lacking polI (Δ*polA*) and expresses a Factor Xa-cleavable His-tagged β clamp (*i.e.* MGHHHHHHHHHHSSGHIEGRHMLD – 1–366 aa of β, where the Factor Xa recognition site is underlined) (Fig. 1).

pTGCP.1.7 encodes all of 5 subunits [*holC* (χ), *holD* (ψ), *holB* (δ′), *holA* (δ) and *dnaX* (τ/γ)] of the *E. coli* clamp-loader complex expressed under the control of T7A1 promoter from a poly cistronic operon [26]. To construct a plasmid that expresses the γ-complex in the absence of the τ-complex, the 3′-terminal fragment of *dnaX* was amplified from pTGCT.1.7 using PCR primers Gamma-front-F and gamma-His-R. The amplicon was digested with DrdI and ligated into the DrdI–AscI sites of pTGCP.1.7 after the vector AscI had been blunt ended with Klenow fragment. Next, a XbaI–PvuI fragment from pTGCP.1.7 (containing a part of β-lactamase, the pMB1 origin, $lacI^q$ and T7A1 promoter) was replaced with a XbaI–PvuI fragment of pHBETA3 (containing a part of β-lactamase, the pSC101 origin, $lacI^q$ and T7 promoter), in order to utilize the same low-copy-number expression system that we employed for the β-clamp. The resulting plasmid, pGCH4, expresses intact χ, ψ, δ′, δ and truncated γ-protein (residues 1–373) with a C-terminal His tag (*i.e.* 1-MSYQV… EVPRQ-373-HHHHHH) under the control of an IPTGinducible T7 promoter (Fig. 1).

2.5. Purification of His-tagged pol V

Strain RW644, harboring pHUC25 and pARAD2 was cultured at 37 °C in 40 ml LB broth (containing 12.5 μg/ml erythromycin, 25 μg/ml ampicillin and 25 μg/ml kanamycin) overnight. The culture was then centrifuged and washed once with and equal volume of LB broth and inoculated to 2 liters LB broth (containing 50 μg/ml ampicillin and 50 μg/ml kanamycin) and incubated at 37 °C with vigorous agitation. When the cell density (A_{600}) reached ~0.4, 4 mg/ml L-arabinose was added to the culture and cells were incubated for 5 h before harvesting by centrifugation at 3800 × *g* for 15 min. The cells were then resuspended in 100 ml of buffer A [50 mM Tris–HCl pH 7.5, 300 mM NaCl, 20 mM imidazole, 10% glycerol] supplemented with Complete EDTA-free protease inhibitor cocktail (Roche Molecular Diagnostics, Alameda, CA) and lysed by sonication, followed by centrifugation at 10,000 × *g* for 20 min. The clarified cell lysate was applied to 1 ml of Ni-NTA agarose (Qiagen, Valencia, CA) and after washing the resin with 6 ml of buffer A, proteins that were retained on the resin were eluted with 3 ml of buffer B [50 mM HEPES, 1 M NaCl, 500 mM imidazole, 20% glycerol, pH 6.8]. Immediately after elution, the pol V containing fractions were loaded onto a Superdex 200 HiLoad 26/60 column (GE Healthcare, Piscataway, NJ) which was pre-equilibrated with buffer C [50 mM HEPES, 1 M NaCl, 2 mM DTT, 20% glycerol, pH 6.8] and proteins were fractionated by isocratic elution with buffer C. About 25 ml of pol V-containing peak fractions were applied to 0.2 g of Bio-Gel HTP hydroxyapatite (Bio-Rad, Hercules, CA), which was pre-equilibrated with buffer C. After washing the resin with 6 ml of buffer C, pol V was eluted in 500 μl-fractions of buffer D [100 mM sodium phosphate, 1 M NaCl, 2 mM DTT, 20% glycerol, pH 6.5]. pol V usually eluted in the second fraction and this was divided into 10 μl aliquots and stored at −80 °C for future use in the *in vitro* replication assays. Current estimates suggest that the enzyme is active for over 1 year under these storage conditions. The concentration of highly purified pol V protein was determined by using a commercially available Bradford Assay [27] (Bio-Rad, Hercules, CA), with BSA of known concentration as a standard.

2.6. Purification of His-tagged β-clamp

DV38(λDE3) harboring pHBETA3 was cultured at 37 °C overnight in 20 ml LB broth containing 15 μg/ml chloramphenicol, 25 μg/ml zeomycin, 20 μg/ml spectinomycin, 12.5 μg/ml erythromycin, 25 μg/ml kanamycin and 100 μg/ml ampicillin. The overnight culture was washed once with LB broth and inoculated into 1 L LB broth containing 100 μg/ml ampicillin and 25 μg/ml kanamycin and incubated at 30 °C with vigorous agitation. When the cell density (A_{600}) reached ~0.4, 1 mM IPTG was added and cells were incubated for 2 h before harvesting by centrifugation at 3800 × *g* for 15 min. The cells were resuspended in 50 ml of buffer E [50 mM Tris–HCl pH 7.5, 100 mM NaCl, 20 mM imidazole, 10% glycerol] supplemented with Complete EDTA-free protease inhibitor (Roche) and lysed by sonication followed by centrifugation at 10,000 × *g* for 20 min. The clarified cell lysate was applied to 500 μl of Ni-NTA agarose (Qiagen) and after washing the resin with 6 ml buffer E, proteins were eluted with 3 ml of buffer F [50 mM Tris–HCl, 100 mM NaCl, 500 mM imidazole, 10% glycerol, pH 7.5]. After adding 100 units of Factor Xa (EMD Biosciences), the solution was dialyzed twice against 500 ml of buffer G [50 mM Tris–HCl pH 8.0, 100 mM NaCl, 5 mM $CaCl_2$] at room temperature for 4–6 h. The dialyzed solution was passed over Ni-NTA agarose (100 μl) and Xarrest agarose (750 μl) (EMB Biosciences, Madison, WI) to eliminate the cleaved N-terminal His-tag and Factor Xa protease. The flow-through fraction was then loaded on a Superdex 200 HiLoad 26/60 column, which was pre-equilibrated with buffer H [50 mM Tris–HCl pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol] and proteins were resolved by isocratic elution with buffer H. Approximately 25 ml of the peak fraction containing the β-clamp was applied to 300 μl of Q sepharose HP (GE Healthcare), which was pre-equilibrated with buffer I and after washing the resin with 6 ml of buffer I [50 mM Tris–HCl pH 7.5, 0.1 mM EDTA, 1 mM DTT, 10% glycerol], the

protein was eluted with a linear NaCl gradient (0–1 M) in buffer I. The peak fraction containing the β-clamp was divided into 20 μl aliquots and stored at −80 °C for subsequent use in the *in vitro* assays. The concentration of β-clamp protein was determined by using the Bio-Rad Protein Assay reagent with BSA as a standard.

2.7. Purification of His-tagged γ complex

DV38(λDE3) harboring pGCH4 was cultured and the expression of the γ complex was induced as for the β-clamp except that the overnight culture was increased to 40 ml and used to inoculate 2 L of LB and cells were induced for 3 h. After harvesting, the cell pellet was resuspended in 100 ml of buffer E supplemented with 2 mM $MgCl_2$ and Complete EDTA-free protease inhibitor. Cells were lysed by sonication followed by centrifugation at 10,000 × *g* for 20 min. The clarified cell lysate was applied to 300 μl of Ni-NTA agarose and after washing the resin with 6 ml of buffer E containing 2 mM $MgCl_2$, proteins were eluted with 3 ml of buffer F containing 2 mM $MgCl_2$. Approximately 3 ml of the eluted protein solution was applied to a Superdex 200 HiLoad 26/60 column, followed by a 300 μl Q sepharose HP column as described above for β-clamp except that buffer H and I contained 2 mM $MgCl_2$ instead of 0.1 mM EDTA. The peak fraction of γ-complex eluting from the Q sepharose column was divided into 20 μl aliquots and stored at −80 °C for subsequent use in the *in vitro* assays. The concentration of β-clamp protein was determined by using the Bio-Rad Protein Assay reagent with BSA as a standard.

2.8. In vivo mutagenesis assays

The ability of our various plasmid constructs to promote muta-genesis *in vivo* was assayed by monitoring spontaneous reversion of the *hisG4*(ochre) allele in RW584 [*hisG4, lexA51*(*Def*), *recA730*, Δ*umuDC*] [28]. Strain RW584 was either transformed with pGB2 (vector) [29], pRW134 (*umuD′C*) [20], pSCD1 (*umuCD′*), pSCD2 (*his-umuCD′*), pSCD2-D101N (D101N, *umuC104* [30] active site mutant), or pSCD2-DEAA (D101A and E102A, *umuC* active site substitutions). Fresh transformants were used for each assay. An overnight culture was grown at 37 °C in 12 ml LB broth containing 25 μg/ml erythromycin and 100 μg/ml spectinomycin. The number of viable cells in the culture was determined by spreading 50 μl of a 10^{-6} dilution of the overnight culture on LB agar plates in duplicate. To measure reversion of the *hisG4* allele, 5 ml (for poorly mutagenic plasmids, *i.e.* pGB2), or 100 μl (for highly mutagenic plasmids like pRW134) were washed twice with PBS to eliminate trace amounts of rich medium and spread on Davis and Mingioloi minimal agar plates [31] in duplicate. The viable colonies growing on the LB agar plates were counted after one day incubation at 37 °C, whilst the histidine mutants arising on the minimal agar plates were counted after 2 days incubation at 37 °C. At least 5 clones were examined for each plasmid. The mutation frequency was calculated by dividing the number of His^+ revertants by the number of living cells. Error-bars represent the standard error of the mean (SEM).

2.9. In vitro TLS assays with pol V in the presence, or absence, of accessory proteins

A 5′-^{32}P labeled 17 mer primer, M13-TT, was annealed to pSOcpd [14], a single-stranded circular plasmid containing a unique a T-T cyclobutane pyrimidine dimer (CPD). The primer anneals to the CPD-containing template such that the 3′ of the primer is located 6 nucleotides upstream of the CPD. Pol V (400 nM) was added to a 10 μl reaction mixture containing 5 mM ATP, 200 μM dNTPs, and 4 nM primed pSOcpd in a 1× reaction buffer [20 mM Tris–HCl pH 7.5, 8 mM $MgCl_2$, 8 mM DTT, 80 μg/ml BSA, 4% glycerol]. When indicated, 200 nM (as tetramer) SSB (Epicentre Biotechnologies, Madison WI), 100 nM β-clamp, 50 nM γ-complex, and 2 μM RecA (New England Biolabs, Ipswich, MA) were added and reactions were incubated at 37 °C for 20 min. Where noted, RecA protein was omitted from the reactions and replaced by 0.125 μM RecA*. In such cases, RecA* was pre-

formed by incubating 8 μM RecA, with 2 mM ATPγS and 0.5 μM 48-mer oligonucleotide in 1× reaction buffer at 37 °C for 10 min prior to mixing with pol V. The resulting pol V Mut complex was then added to the replication assays. Reactions were terminated by adding 10 μl of 2× loading buffer [97% formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue]. The products were heat-denatured and immediately resolved by denaturing PAGE (8 M urea, 12% acrylamide), followed by visualization on a Fuji image analyzer FLA-5100.

2.10. Pol V-dependent primer extension assays under "single-hit" conditions

pSO, a single-stranded circular plasmid without a lesion, was constructed according to Karata et al. [14] by using UTTC48P instead of a lesion containing primer, TTC48P and pSO was annealed with 5′-^{32}P labeled primer pSOcpd-2 as described [14]. Reactions were performed essentially as described above, dNTPs were omitted from the pre-reaction mixture. Reactions were started by adding 200 μM dNTPs ±1 mg/ml heparin and stopped by addition of loading buffer after incubation for 20 s, 40 s, 1 min, 2 min, 4 min, 8 min, or 16 min. For the control experiment, heparin was added to the RecA* before combining with the pol V and adding to the remaining reaction mixture. Replication products were analyzed as above.

2.11. Processivity of pol V Mut

RecA was incubated in 1× reaction buffer for 10 min at 37 °C with the 30-mer biotinylated oligomers linked to streptavidin-coated agarose resin in the presence of ATPγS to form RecA* [32]. Pol V Mut was obtained by incubating pol V with the RecA* for 10 min at 37 °C. The resulting complex was recovered from the supernatant by centrifugation and immediately used in the processivity assays, as the isolated pol V Mut complex exponentially loses its activity. Once deactivated, pol V Mut is reactivated with new RecA* [32]. Reaction were performed as described above, except no trap was used, because pol V Mut deactivates after every round of primer extension [32] and as a consequence, heparin has no effect on pol V Mut processivity (data not shown).

3. Results

3.1. Low level expression of UmuC combined with high level expression of UmuD′ results in high yields of soluble pol V

Previously published protocols describing the purification of $UmuD'_2C$ require very large cultures (>100 L) and as many as seven [13] or eight [10] chromatographic steps and result in low yields of the purified complex. We therefore set out to develop a novel strategy to overproduce soluble $UmuD'_2C$. To this end, we explored a variety of strategies that might help increase the recovery of soluble UmuC.

First, we found that a hexahistidine (MHHHHHH) tag to the N-terminus of the UmuC protein greatly facilitated downstream purification. This tag cannot be removed from the recombinant protein, but does not apparently interfere with the biological activity of UmuC (see below). We also generated recombinant versions with longer N-terminal His-tags with protease cleavage sites or GST-tags, but these proteins exhibited minimal solubility and were not characterized further.

Next, we tried the strategy of co-expressing $UmuD'_2C$ with the molecular chaperones DnaK–DnaJ–GrpE (DnaKJE), or DnaKJE with GroESL since these proteins were previously shown to help UmuC fold correctly *in vitro* and *in vivo* [33-35]. However, while there was a modest increase in solubility of UmuC under these expression conditions, the overexpressed

chaperones were much more abundant than $UmuD'_2C$ and they proved to be difficult to eliminate from the $UmuD'_2C$ preparation. As a consequence, we discontinued this approach.

While exploring alternative approaches to express soluble $UmuD'_2C$, we serendipitously identified two expression plasmids that gave relatively good recovery of soluble $UmuD'_2C$. DNA sequence analysis of these plasmids revealed the unintended loss of either the promoter, or the ribosome-binding site (RBS) for *umuC* on both plasmids. As a consequence, rather than over-expressing UmuC protein (as we had hoped), both plasmids expressed UmuC at low basal levels.

We therefore considered the possibility that UmuC might be most soluble when expressed at low, rather than high intracellular levels. To test this hypothesis, we constructed various plasmids that express low levels of UmuC. The plasmids which gave the highest levels of soluble $UmuD'_2C$ were pHUC25 and pARAD2 (Table 1 and Fig. 1). pHUC25 is a low-copy-number plasmid with a pSC101 origin of replication. It expresses His-tagged UmuC upstream of UmuD′. This synthetic operon has a good RBS, but no promoter. Since the *umu* genes are transcribed in the same direction as the plasmid encoded $lacI^q$, we assume that His-UmuC (and UmuD′) is expressed at low levels by transcriptional read-through from the upstream $lacI^q$ promoter. pARAD2 is a compatible high-copy-number plasmid, with a pUC origin of replication. It expresses UmuD′ at high levels from an L-arabinose-inducible promoter.

The two plasmids were expressed in *E. coli* strain RW644, a derivative of BL21(λDE3) with deletions in *polB, dinB* and *umuDC* (Table 1). The strain was cultured until early-mid log phase and only UmuD′ expression (from pARAD2) was induced by the addition of L-arabinose. As expected, there was no obvious increase in expression of UmuC in the induced whole-cell extract (Fig. 2A), but there was visible induction of a protein of the size of UmuD′. Cells were lysed by sonication and applied to a Ni-NTA agarose column. Proteins retained on the column were eluted in a buffer containing 500 mM imidazole 1 M NaCl and 20% glycerol. Quite remarkably, this fraction was highly enriched with UmuC and UmuD′ (Fig. 1A, track E). The complex was further purified by gel filtration to remove minor levels of contaminants. The peak fractions from the Superdex 200 column were concentrated on a hydroxyapatite column and highly purified $UmuD'_2C$ was eluted in a buffer containing 100 mM sodium phosphate (Fig. 2B). 1 M NaCl and 20% glycerol were also added to the elution buffer, since these reagents help prevent aggregation and precipitation of concentrated $UmuD'_2C$ [6].

Of particular significance, is the fact that our novel three-step protocol to purify pol V reduced the time required to purify the enzyme from weeks, to just 1–2 days. Furthermore, the yield was dramatically increased from 1 to 2 mg per 100 L (or more) of starting culture, to ~1 mg per 4 L starting culture.

3.2. His-tagged pol V is highly active in vivo

We were obviously concerned that the seven extra amino acids (MHHHHHH) added to the N-terminus of UmuC to facilitate purification, might somehow affect its biological functions *in vivo* and *in vitro*. We therefore assayed the ability of the His-tagged UmuC protein to promote spontaneous SOS mutagenesis *in vivo* in the *E. coli* strain, RW584 [*hisG4, lexA*(Def), *recA730*]. On the *E. coli* chromosome, the *umuD* and *umuC* genes are arranged in an operon with *umuD* upstream of *umuC*, and the two genes overlap by one nucleotide. In our expression constructs, His-tagged *umuC* is located upstream of *umuD* and the genes are separated by nearly thirty base-pairs. As a consequence, we also generated a plasmid in

which native (untagged) *umuC* was placed upstream of *umuD′* and compared it to *umuD′C* in its normal operon arrangement.

As can been seen in Fig. 3, *umuD′C* (pRW134) exhibited ~100-fold higher mutator activity than the vector (pGB2). Plasmids in which the *umu* operon arrangement was reversed [pSCD1 (*umuC* and *umuD′*) and pSCD2 (*His-umuC* and *umuD′*)], showed indistinguishable activity from pRW134 regardless of the His-tag. In contrast, two *umuC* mutants in which the catalytic active site residues were changed (pSCD2-D101N and pSCD2-DEAA), exhibited greatly reduced, or minimal mutagenesis promoting activity (Fig. 3). These results suggest that the His-tag at the N-terminus of UmuC has negligible effect on its activity *in vivo*.

3.3. Purification of E. coli β-clamp and γ-complex free from contaminating polymerases and exonucleases

When we assayed pol V's activity *in vitro*, we discovered that many of the commercially available preparations of *E. coli* RecA and SSB were contaminated with DNA polymerase(s) and/or exonucleases. The RecA and SSB proteins used in our current studies were purchased from New England Biolabs and Epicentre Biotechnologies, respectively and were free from any polymerase and/or exonuclease contaminants (see below).

We also discovered that some of our previous preparations of *E. coli* β-clamp and γ-complex were also contaminated with trace amounts of a DNA polymerase. As a consequence, we developed strategies to overproduce and purify the β-clamp and γ-complex free of contaminants. To facilitate our goal, we utilized an *E. coli* strain, DV38, lacking DNA polymerases I, II, IV and V (Table 1) [18]. The strain was lysogenized with λDE3 and the β-clamp and five-subunit γ-clamp loader complex was expressed from a low-copy-number plasmid with an IPTG inducible promoter (Fig. 1).

To facilitate purification of the β-clamp, we constructed plasmid pHBETA3, which added a His-tag at the N-terminus of the β-clamp. The N-terminal extension included a cleavable Factor Xa site located five amino acids upstream of the normal N-terminus of β. Using this construct, His-tagged β-clamp was induced and captured by Ni-NTA agarose (Fig. 4A). The His-tag was cleaved by Factor Xa during dialysis and the His-tag and Factor Xa were removed from the preparation by Ni-NTA and Xarrest agarose. The β-clamp was further purified *via* Superdex 200 gel filtration and Q-sepharose anion exchange chromatography allowing us to readily purify ~500 μg of β-clamp that is free from contaminating DNA polymerase/exonuclease activities from 1 L starting culture (Fig. 4A).

The γ-clamp loader complex was purified from the *E. coli* strain DV38(λDE3), harboring pGCH4 (Table 1). pGCH4 is a low-copy-number plasmid derivative of pTGCP.1.7 [26]. The 3′ end of the *dnaX* gene was initially altered to express a recombinant γ-protein with a His-tag at its C-terminus. However, the protein bound poorly to Ni-NTA agarose, possibly because the C-terminus of the γ-protein is disordered. We therefore deleted the disordered region of the γ-protein and instead added the C-terminal His-tag to the truncated γ-protein (residues 1–373), which retains full clamp-loading activity [36]. The recombinant γ-complex was purified free of polymerase/exonuclease contaminants by Ni-NTA agarose, Superdex 200 and Q-Sepharose chromatography (Fig. 4B). Approximately 100 μg of the highly pure γ-complex could be readily obtained from 2 L of starting culture.

3.4. pol V-dependent translesion DNA synthesis in vitro

Using highly purified pol V, β-clamp and γ-complex together with commercially available RecA and SSB, we determined the reaction conditions for optimal translesion DNA synthesis. The substrate for these studies was pSOcpd [14], a 3.0 kb circular, single-stranded

template containing a single cyclobutane pyrimidine dimer (CPD) (Fig. 5A). As noted above, we ensured that none of the accessory factors assayed here contain any contaminating polymerase/exonuclease activities and this is depicted in Fig. 5B, where no primer extension or degradation is observed in the presence of 2 μg of each protein.

Where noted, tetrameric SSB was added at a final concentration of 200 nM. This is sufficient to completely cover the 3.0 kb single-stranded template, assuming that a tetramer occupies 65 nucleotides [37]. Reactions also contained 2 μM RecA which was either directly added to the reaction mixture, or in a form of nucleoprotein filament (RecA*). When added directly to the reaction, 2 μM RecA is sufficient to cover approximately half of the 3.0 kb DNA template, assuming one RecA protomer occupies 3 nucleotides [38]. Under these conditions, the RecA protein is tacitly assumed to form a RecA filament on the single-stranded pSOcpd template being copied. Since the reaction contains multiple template molecules, *trans* activation of pol V by RecA* occurs under these conditions [39]. However, as previously discussed [40], *cis* activation of pol V by RecA* bound to the template being copied cannot be formally excluded. In contrast, when RecA* was pre-formed on an undamaged 48-mer oligonucleotide in the presence of ATPγS and added to the reaction at a concentration of 0.125 μM (which corresponds to 2 μM RecA), it is constrained to act solely *in trans* to activate pol V.

As shown in Fig. 5C, track 2, pol V *per se* exhibits extremely weak polymerase activity in the absence of accessory factors. However, when added individually to the reaction, SSB (track 3), β-clamp (track 4), RecA (track 6) or *trans*-RecA* (track 10) all stimulated pol V activity on undamaged DNA, but did not facilitate TLS of the CPD. Certain combinations of these accessory factors (*i.e.* SSB plus β-clamp [track 5]) further enhanced pol V activity, but again, no detectable TLS was observed. In contrast, TLS was observed in the presence of the β-clamp and RecA (track 8) or *trans*-RecA* (track 12 and 13). The effect of SSB depended on the mode of RecA addition. In the presence of *trans*-RecA*, SSB greatly enhanced the activity of pol V (tracks 11 and 13). But when RecA protein was added directly to the reaction, SSB actually inhibited pol V (track 9), presumably by interfering with RecA nucleoprotein filament formation [41].

Replication of damaged templates in the presence of the β-clamp and RecA (track 8) appears to be distributive with a strong pause site prior to the CPD. However, when RecA was replaced by RecA*, TLS appears to be much more processive (track 12 and 13), with minimal pausing at the site of the CPD and replication products of several hundred nucleotides in length. Therefore, robust pol V-dependent TLS occurs in the presence of RecA* (activated *in trans*), the β-clamp and SSB protein (*c.f.* track 8 *vs.* 13).

3.5. Processivity of pol V in the presence of accessory factors

Since in the presence of β-clamp and *trans*-RecA*, pol V synthesized very long replication products (Fig. 5C, tracks 12 and 13), we were interested in determining the processivity of pol V under these assay conditions. To do so, we followed pol V-dependent primer extension over a period of time in the presence of heparin, which quenches any polymerase molecules that dissociate from the template [42]. Such an approach allows us to assay the extent of pol V-dependent replication in just one “polymerase-binding event”. As expected, addition of heparin to the reaction prior to pol V completely inhibited primer extension (Fig. 6, lane 1). In contrast, however, when heparin was added after pol V, but at the onset of the reaction, the synthesized replication products became increasingly longer over the 16 min assay (Fig. 6, lanes 2–8). These data revealed that in the presence of the β-clamp, SSB and *trans*-RecA*, pol V appears to be processive, with >200 nucleotides synthesized per single-binding event. This is in dramatic contrast, to previous reports indicating a processivity of ~6–25 nucleotides per single binding event [10,12,43,44]. However, the fact that replication

products increase over at least 16 min indicates that the enzyme is very slow at catalysis. Indeed, we estimate that the velocity of pol V is ~0.3–1 nucleotide incorporated per second, which is in dramatic contrast to pol III, which is believed to synthesize ~600 nucleotides per second [45].

3.6. Requirements for processive synthesis by pol V Mut (pol V·RecA·ATP)

The experiments described above, indicate that pol V exhibits a considerable degree of processivity in the presence of accessory factors. It appears that a major determinant of processivity is the presence of *trans*-activating RecA* (*c.f.* Fig. 5C, tracks 8 *vs.* 12). Jiang et al. [32] have previously shown that a protomer of RecA (plus ATP), is transferred from RecA* to pol V to generate pol V Mut (pol V·RecA·ATP). Pol V Mut rapidly loses activity after one round of DNA synthesis, but active pol V Mut can be regenerated by replacing the RecA·ATP in the deactivated pol V Mut with fresh RecA·ATP from *trans*-activating RecA* [32]. While the experiments described above (Fig. 6) reflect products generated from a single polymerase-binding event, they were nevertheless performed in the presence of an excess of *trans*-RecA*. We were therefore interested in determining the processivity and cofactor requirements for pol V Mut directly. To do so, we first isolated pol V Mut as described [32]. Primer extension assays were then performed as described above, in the absence of RecA*, but in the presence or absence of β-clamp and/or SSB. As seen in Fig. 7A, in the presence of the β-clamp and SSB protein, pol V Mut is a processive, but slow enzyme, with replication products extending 12–15 nucleotides in 20 s after initiation of the reaction, to at least several hundred nucleotides by 16 min. Our data therefore indicate that like pol V in the presence of excess *trans*-activating RecA* (Fig. 6), pol V Mut can remain bound to the primer terminus for a considerable period of time. Omission of the β-clamp from the reaction did not alter the pattern of elongation products observed at earlier time points, but primer extension was completed by ~2 min after reaction initiation and as a result, final replication products were significantly shorter (Fig. 7B, 4–16 min). From this, we conclude the following: in the presence of SSB, the β-clamp is required for extended pol V Mut synthesis; in the absence of β-clamp, pol V Mut can remain stably bound to a primer terminus for a period no longer than 2 min and in a time-dependent manner synthesize anywhere between 1 and ~30 nucleotides per binding event. A substantially different termination pattern was observed in the absence of SSB protein (Fig. 7C). Despite the fact that the assays contained β-clamp, there was very little primer extension in the absence of SSB, and minimal extension over time in the presence of low levels of SSB (Fig. 7D). Under these conditions, SSB may stimulate pol V Mut by simply decreasing the secondary structure of the long single-stranded template strand being copied. However, since SSB has been shown to physically interact with UmuC [9], the stimulation more likely occurs *via* a direct protein-protein interaction with pol V Mut that helps target the polymerase to the nascent primer terminus.

4. Discussion

4.1. A novel approach to purify E. coli pol V

E. coli pol V ($UmuD'_2C$) has previously proven notoriously difficult to purify. Here, we report the development of a novel strategy to obtain large quantities of soluble pol V from small-scale cultures. The key to our success, was that the UmuC protein was His-tagged at its N-terminus and rather than overproducing His-UmuC, the recombinant protein was expressed at low basal levels in the presence of copious amounts of UmuD′. The purification protocol only requires three chromatographic steps, and can be completed in just one day. Using this protocol, approximately 1 mg of highly purified pol V can be obtained from ~4 L of bacterial culture (Fig. 1). We anticipate that the availability of large quantities of highly purified pol V will, therefore, provide researchers an excellent opportunity to

expand our understanding of the mutagenic process through future biochemical and structure-function studies on pol V and mutant variants.

4.2. Activity of pol V in the presence of accessory cofactors

When we assayed the ability of pol V to extend a primer bound to a circular-single-stranded template containing a CPD, we were surprised by the fact that the catalytic activity of pol V (on undamaged DNA) and its ability to promote TLS, varied considerably depending upon the addition of the specific accessory factor and how/when it was added to a reaction. The addition of β-clamp always improved primer extension, but by itself did not facilitate TLS of the CPD lesion (Fig. 5C, track 4). Indeed, TLS was only observed in the combined presence of RecA and β-clamp (Fig. 5C, tracks 8 and 12), and such observations are consistent with the genetic requirements for both proteins in TLS [46,47]. The effect of RecA and SSB on pol V's overall catalytic activity, processivity, and ability to facilitate TLS was more complex. In reactions where RecA protein was added directly to the reaction mix, pol V exhibited a combination of distributive and weakly processive primer extension activity (Fig. 5C, track 8), presumably because RecA* filaments formed on the template strand being copied physically impede pol V [48]. Furthermore, this activity was suppressed by the addition of SSB presumably due to the interference with RecA* formation (Fig. 5C, track 9). In contrast, in the presence of β-clamp and RecA* formed *in trans*, pol V is highly active and bypasses the CPD with minimal pausing and in doing so, generates replication products of at least several hundred nucleotides in length (Fig. 5C, track 12). In addition, and in contrast to the situation when RecA was added to the reaction directly, SSB clearly *stimulated* primer utilization, catalytic activity and TLS functions of pol V (Fig. 5C, track 13).

5. Conclusions

We have been able to significantly simplify the purification of soluble $UmuD'_2C$ providing us with substantial quantities of ultra-pure pol V required for detailed biochemical characterization. It has also allowed us to resolve the controversy around cofactor requirements for optimal polymerase performance *in vitro*. We show that when pol V is activated *in trans* by a preformed RecA* filament, replication of undamaged and CPD-containing circular DNA templates in the presence of β/γ complex and SSB protein proceeds with much higher efficiency and processivity than previously reported.

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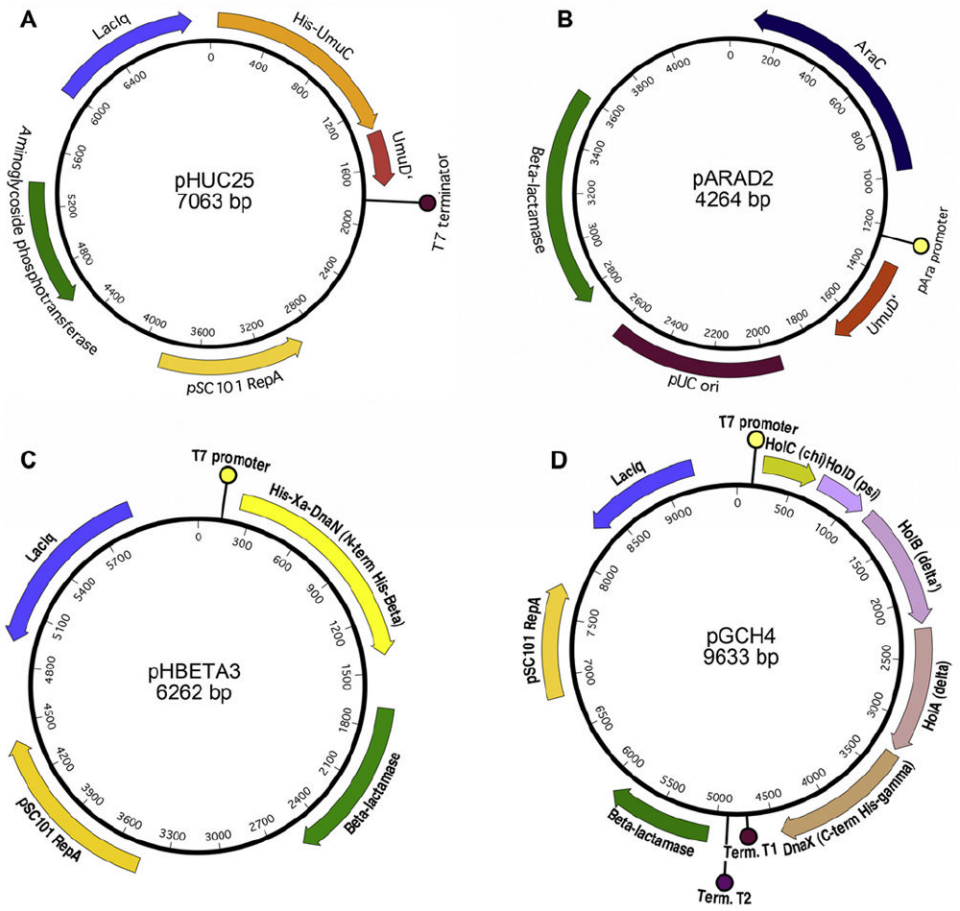

Fig. 1.
Plasmid maps of pHUC25 (A), pARAD2 (B), pHBETA3 (C) and pGCH4 (D).

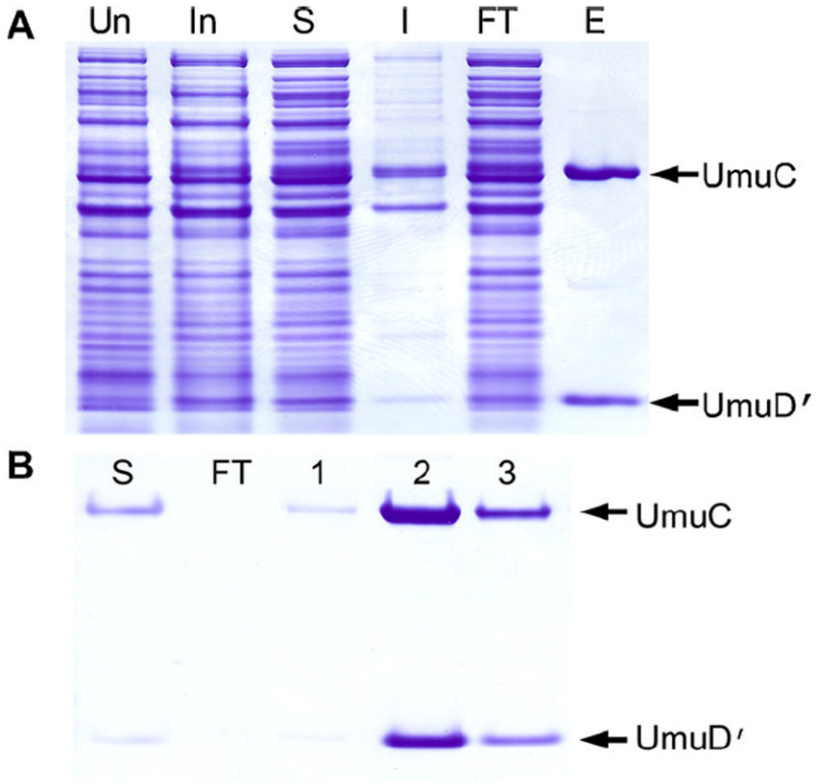

Fig. 2.

Purification of pol V. (A) SDS-PAGE gel of His-UmuC/UmuD′ purification using Ni-NTA chromatography. Lanes labeled *Un* and *In* are uninduced or induced whole-cell extracts, respectively. Lanes *S* and *I* are the soluble and insoluble fractions respectively. Lanes *FT* and *E* are the flow-through fractions and fractions eluted from Ni-NTA agarose, respectively. (B) SDS-PAGE gel of pol V purified/concentrated by hydroxyapatite chromatography. Lane *S* is the pooled Superdex 200 fractions. Lane *FT* is the flow-through fraction. Lanes labeled 1, 2, 3, are eluted fractions containing highly purified pol V.

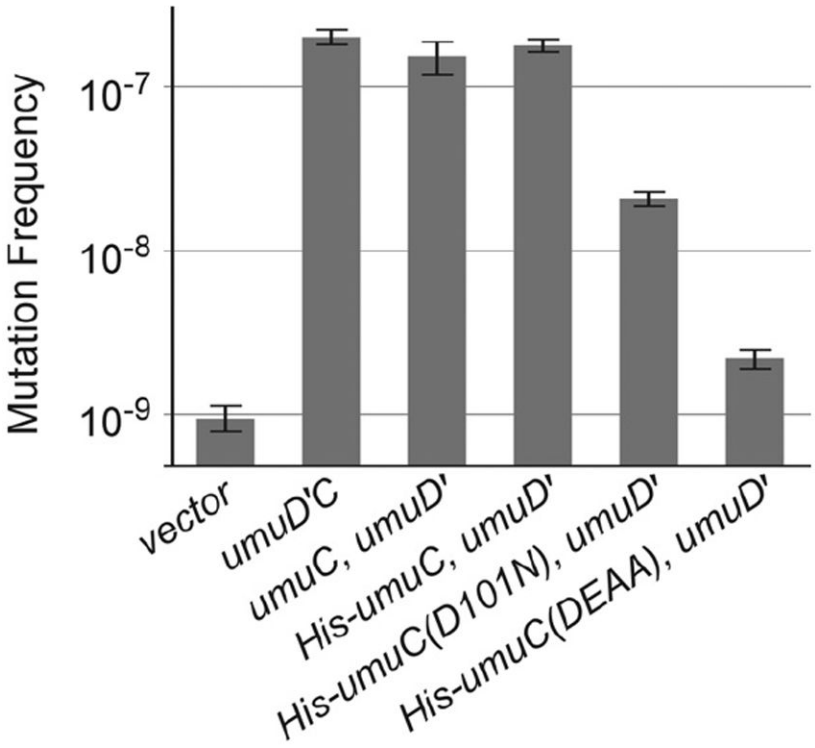

Fig. 3.

In vivo mutagenesis promoting activity of His-pol V. Spontaneous SOS mutagenesis was followed by assaying reversion of the *hisG4*(*ochre*) allele in *E. coli* strain RW584 (*recA730, lexA*(Def) Δ*umuDC*) and was assayed in the presence of pGB2 (vector), pRW134 (*umuD´C*), pSCD1 (*umuCD´*), pSCD2 (*his-umuCD´*), pSCD2-D101N (D101N mutant), or pSCD2-DEAA (D101A, E102A mutant). See Section 2 for more details. Error bars represent the standard error of the mean (SEM).

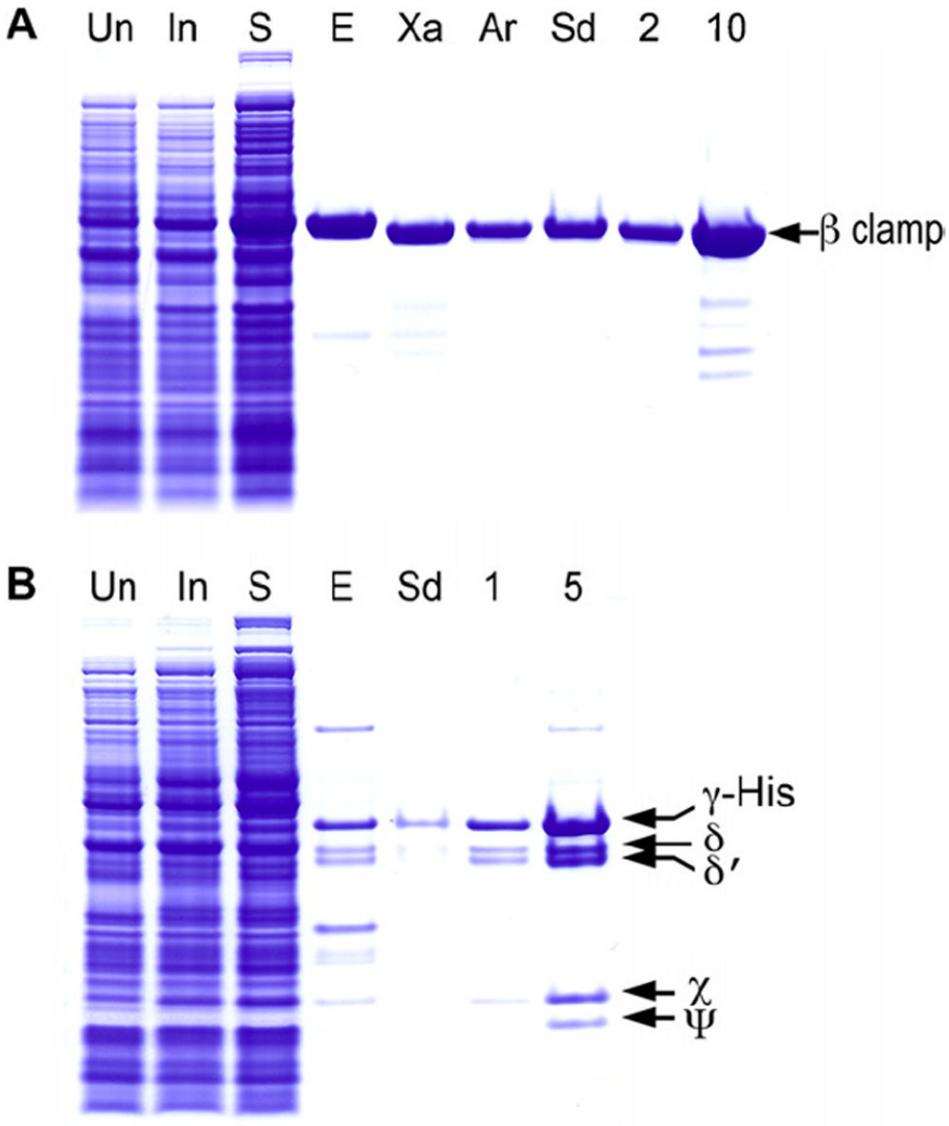

Fig. 4.

β/γ complex purification. (A) Purification of β-clamp. Lanes *Un* and *In* are uninduced and induced whole cell extracts respectively. Lane *S* is the soluble fraction. Lane *E* is the fraction obtained after elution from Ni-NTA agarose. Lane *Xa* is the fraction obtained after treating with Factor Xa protease. Lane *Ar* is the flow-through fraction obtained from the Ni-NTA agarose and Xarrest agarose columns. Lane *Sd* is the peak fraction from the Superdex 200 column. Lanes labeled *2* and *10* are 2 and 10 μg, respectively, of the final preparation that eluted from the Q sepharose column. (B) Purification of γ-complex. Lanes *Un* and *In* are uninduced and induced whole cell extracts respectively. Lane *S* is the soluble fraction. Lane *E* is the fraction obtained after elution from Ni-NTA agarose. Lane *Sd* is the peak fraction from the Superdex 200 column. Lanes labeled *1* and *5* are 1 and 5 μg, respectively, of the final preparation that eluted from the Q sepharose column.

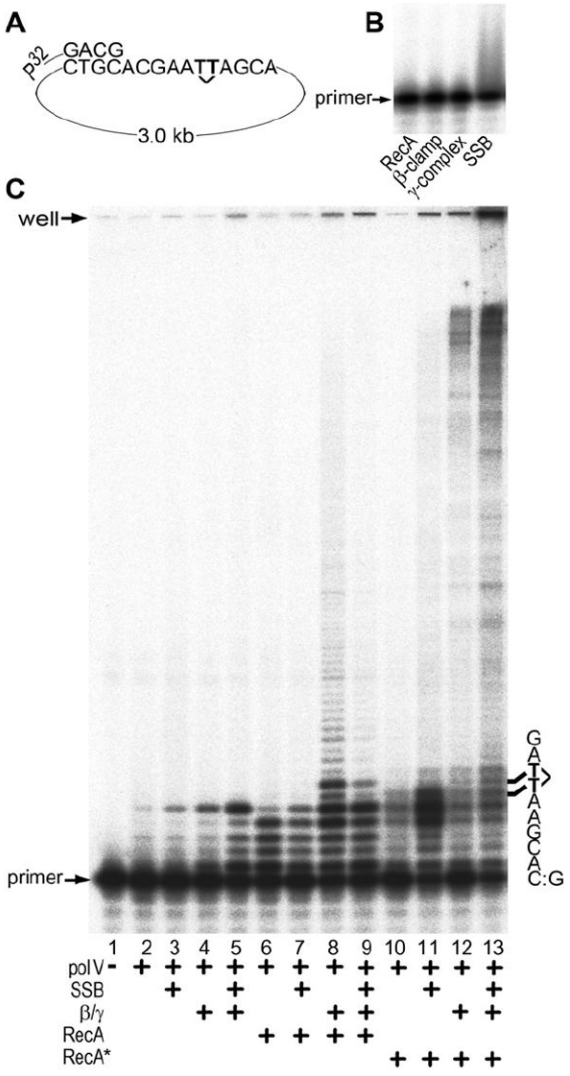

Fig. 5.

Biochemical characterization of pol V. (A) Cartoon of the lesion-containing 3.0 kb DNA template used in these studies. The nucleotide sequence surrounding the single CPD is shown. The 3′ end of the ^{32}P radiolabeled primer is located 5 nucleotides from the CPD and these assays are therefore considered “running start” replication assays. (B) Analysis of accessory factors for contaminating polymerase/exonuclease activities. Each protein (2 μg) was added to the reaction mixture of 1× reaction buffer, 200 μM dNTPs and 10 nM 48-mer oligonucleotide template which was annealed with 5′-^{32}P labeled 17-mer primer and incubated at 37 °C for 30 min. As can be clearly seen, all preparations are free of contaminating polymerase/exonuclease activities. (C) *In vitro* activity of His-pol V in the absence or presence (+) of accessory proteins were carried out as described in Section 2.9. 400 nM of pol V, 200 nM of SSB, 100 nM β-clamp, 50 nM γ-complex, 2 μM RecA or 2 μM RecA* (as the concentration of RecA protein) was used where indicated. Lane 1 depicts the radiolabeled primer in the absence of proteins.

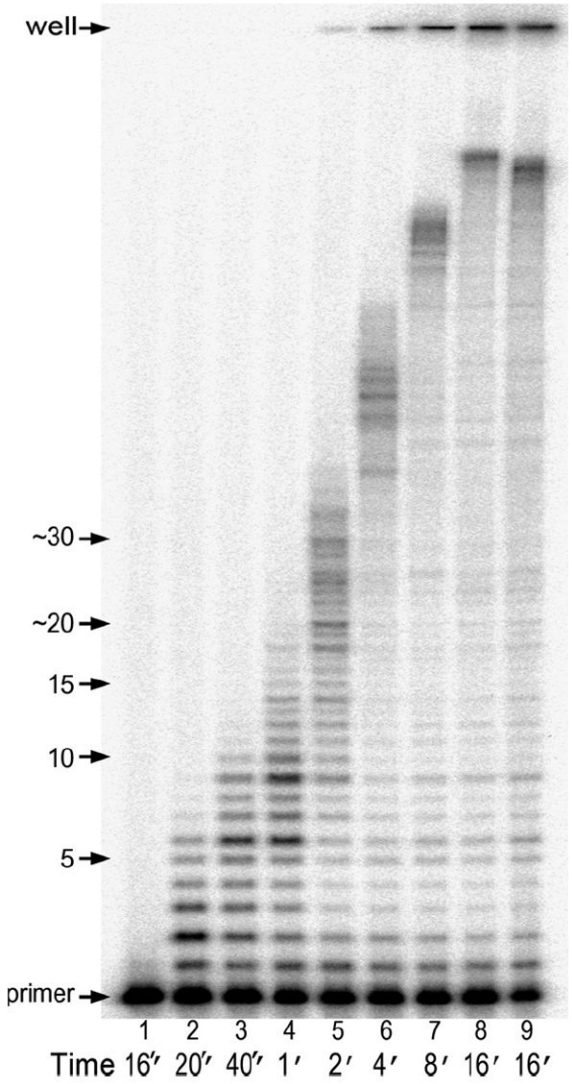

Fig. 6.

Processivity of pol V in the presence of Heparin. Experiments were performed as in Fig. 5C, lane 13, except dNTPs were added last to initiate the reactions and heparin was used to prevent pol V from rebinding. Lane 1, heparin was added prior to the addition of pol V. No synthesis is observed, indicating that the heparin is acting as a molecular trap. Lanes 2–8: pol V-dependent DNA synthesis on an undamaged template was assayed in the presence of heparin and the reaction stopped at the indicated time. Lane 9, replication in the absence of heparin.

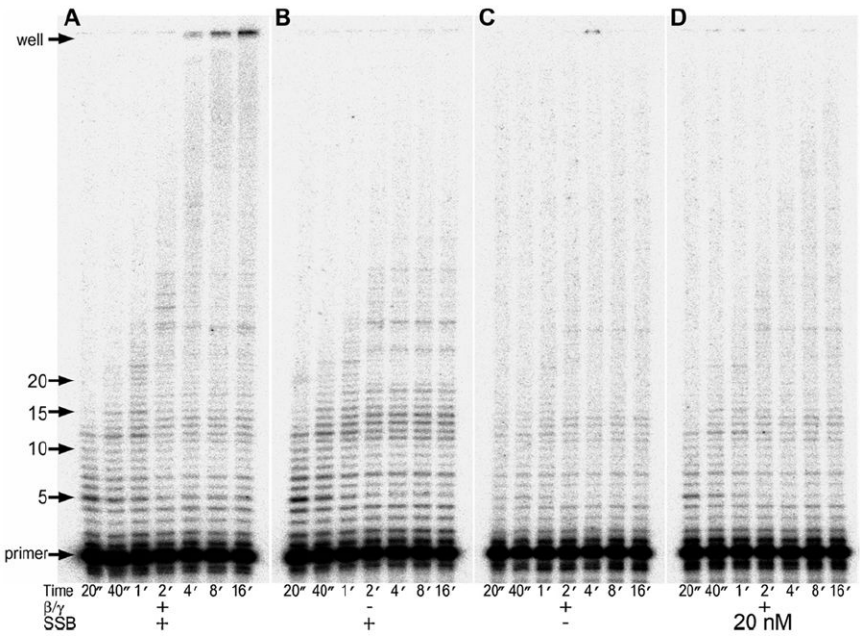

Fig. 7.

Processivity of pol V Mut in the presence or absence of β-clamp and SSB. (A) RecA was incubated with the biotinylated oligomers linked to streptavidin-coated agarose resin. Resulting RecA* was mixed with pol V to produce pol VMut which was recovered from the supernatant by centrifugation and used in the processivity assays as described in Fig. 5, except no heparin was used. (B) Reactions were carried out as above, except β/γ complex was omitted. (C) Reactions were carried out as above, except SSB protein was omitted. (D) Reactions were performed as above, except that SSB protein was used at 10 times lower concentration (20 nM).

Table 1

Strains and plasmids.

Strain	Relevant genotype	Source
RW644	BL21(λDE3), *ΔpolB1::Ωspec, ΔdinB61::ble, ΔumuDC596::ermGT*	This work
RW584	*lexA51*(Def), *recA730, ΔumuDC596::ermGT, hisG4*	[28]
DV38	*ΔpolA::kan, ΔpolB1::Ωspec, ΔdinB61::ble, ΔumuDC596::ermGT*	[18]
DV38(λDE3)	DV38 lysogenized with λDE3	This work

Plasmid	Relevant characteristics	Resistance marker	Source
pBB528	pSC101 ori, *laqI^q*	Chloramphenicol	[22]
pRW134	pSC101 ori, *umuD′C*	Spectinomycin	[20]
pEC48	pET11d, *umuD′*	Ampicillin	[21]
pHUC25	pSC101 ori, *laqI^q, His-umuC, umuD′*	Kanamycin	This work
pBAD24	pMB1 ori, P_{BAD} promoter, *araC*	Ampicillin	[23]
pARAD2	pUC ori, *umuD′*	Ampicillin	This work
pGB2	pSC101 ori	Spectinomycin	[29]
pSCD1	pSC101 ori, *umuC, umuD′*	Spectinomycin	This work
pSCD2	pSC101 ori, *His-umuC, umuD′*	Spectinomycin	This work
pRW392	pMB1 ori, P_{tac} promoter *umuD′umuC* (D101N)	Ampicillin	[7]
pSCD2-D101N	pSC101 ori, *His-umuC* (D101N), *umuD′*	Spectinomycin	This work
pSCD2-DEAA	pSC101 ori, *His-umuC* (D101A, E102A), *umuD′*	Spectinomycin	This work
pHBETA3	pSC101 ori, *His-dnaN*	Ampicillin	This work
pSJS9	pMB1 ori, *recF, dnaN*	Ampicillin	[25]
pTGCP.1.7	pMB1 ori, *holC, holD, holB, holA, dnaX*	Ampicillin	[26]
pGCH4	pSC101 ori, *holC, holD, holB, holA,Δ-Cterm dnaX-His*	Ampicillin	This work

Table 2

List of oligonucleotide primers used in this study.

NdeI-UmuC-F:
5′-TGG AAC ATA TGC ACC ATC ATC ACC ACC ACA TGT TTG CCC TCT GTG ATG-3′
XhoI-UmuC-R:
5′-GCC GCT CGA GTT ATT TGA CCC TCA G-3′
UmuD′-F:
5′-TAA TCT CGA GTA ACT TTA AGA AGG-3′
UmuD′-Xho-R:
5′-GGT GCT CGA GTG CGG CCG TTA GCG CAT CGC CTT AAC G-3′
UmuD′-R:
5′-AGG GCG GCC GTT AGC GCA TCG CCT TAA CG-3′
AR127:
5′-CAT ATG CTC GAT ATG AAA TTT ACC GTA GAA CGT GAG C-3′
AR128:
5′-AAG CTT TGT TGC GGA AAT CGC GGA TCA ACA AGC GGG-3′
Gamma-front-F:
5′-GAC GAC CGA TCC ACA GA-3′
Gamma-His-R:
5′-TTT AAT GGT GGT GGT GAT GGT GCT GTC GTG GCA CTT CTG G-3′
M13-TT:
5′-GAT CGA TGG TAC GGA CG-3′
pSOcpd-2:
5′-CGA CGG TAT CGA TAA GC-3′
UTTC48P:
5′-TCG ATA CTG GTA CTA ATG ATT AAC GAC TTA AGC ACG TCC GTA CCA TCG-3′