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The DNA polymerase III holoenzyme contains γ and is not a trimeric polymerase

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ABSTRACT

There is widespread agreement that the clamp loader of the *Escherichia coli* replicase has the composition $DnaX_3\delta\delta'\chi\psi$. Two DnaX proteins exist in *E. coli*, full length τ and a truncated γ that is created by ribosomal frameshifting. τ binds DNA polymerase III tightly; γ does not. There is a controversy as to whether or not DNA polymerase III holoenzyme (Pol III HE) contains γ. A three-τ form of Pol III HE would contain three Pol IIIs. Proponents of the three-τ hypothesis have claimed that γ found in Pol III HE might be a proteolysis product of τ. To resolve this controversy, we constructed a strain that expressed only τ from a mutated chromosomal *dnaX*. γ containing a C-terminal biotinylation tag (γ-C_{tag}) was provided in *trans* at physiological levels from a plasmid. A 2000-fold purification of Pol III* (all Pol III HE subunits except β) from this strain contained one molecule of γ-C_{tag} per Pol III* assembly, indicating that the dominant form of Pol III* in cells is $Pol\ III_2\tau_2\ \gamma\delta\delta'\chi\psi$. Revealing a role for γ in cells, mutants that express only τ display sensitivity to ultraviolet light and reduction in DNA Pol IV-dependent mutagenesis associated with double-strand-break repair, and impaired maintenance of an F' episome.

INTRODUCTION

The DNA polymerase III holoenzyme (Pol III HE) from *Escherichia coli* serves as a prototype for all cellular polymerases (for a review, see (1)). It is tripartite, containing a replicative polymerase (Pol III core αεθ), a sliding clamp processivity factor (β_2) and a clamp loader, the DnaX complex ($DnaX_3\delta\delta'\chi\psi$; $DnaX_{cx}$). The *dnaX* gene expresses two proteins, γ and τ (2–4). γ is expressed through programmed translational frameshifting, producing a shortened DnaX protein (47 kDa) that contains the domains required for the ATP-dependent loading of β_2 on DNA and interaction sites for binding additional DnaX subunits and δ and δ′ (5,6). The full length *dnaX* translation product τ (71 kDa) also contains domains that interact with the replicative helicase ($DnaB_6$) and the α subunit of the Pol III core (7,8). The $DnaX_{cx}$ plays a central functional and organizational role at the replication fork, linking the leading and lagging strand polymerases and the replicative helicase (9,10). τ-containing $DnaX_{cx}$ chaperones Pol III α onto nascently loaded β_2, conferring a kinetic advantage necessary to sustain the physiological rate of DNA replication (11,12). τ-containing $DnaX_{cx}$ may serve as the sensor that recognizes the availability of a new primer at the replication fork, forcing the lagging strand polymerase to cycle to the new primer by a signaling mechanism (13).

The composition of the $DnaX_{cx}$ has remained controversial. There is now agreement that it contains three copies of DnaX and one each of δ, δ′, χ and ψ (14,15). A model was put forward (14) that Pol III HE contains two copies of τ and one of γ, based on the finding that at least two copies of τ are required to couple the leading and lagging strands (10) and the fact that a subassembly containing two Pol IIIs and two τs (Pol III') has been isolated from cells (16). All preparations of chromosomally expressed Pol III HE isolated from cells contain both τ and γ. Pol III HE immunoprecipitated by a monoclonal antibody directed against the unique C-terminal domains of τ co-precipitated γ by its association with the same complex (17). Concern about the origin of γ was raised by the finding that τ could be proteolyzed to a protein of nearly the same size as γ by the OmpT protease (18). However, OmpT is in the periplasmic space and τ is only exposed to it after cell lysis. We showed, by avoiding dialysis to low salt in crude extracts, that this

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artifactual proteolysis could be avoided. With this finding, we constructed an *ompT* strain that was used for all subsequent purifications (19). $DnaX_{cx}$ containing only τ could be purified from such strains by using a mutant form of *dnaX* that could not frameshift, demonstrating that there is not another protease in the cell that could cleave τ to a γ-sized product. Additional confidence was derived from the finding that γ occupied a unique position within the $DnaX_{cx}$, a situation that would not be expected if γ was generated in Pol III HE by artifactual proteolysis (20).

Subsequent to these findings, proposals have been made that Pol III HE contains three copies of τ and none of γ. The first proposal derived from assembling Pol III HE *in vitro* in the absence of γ (21). It was proposed that the triple DNA polymerase replicase assembled could be the physiological form. However, since this enzyme was assembled under conditions where γ was omitted and no attempt was made to isolate Pol III HE from cells, evidence for the physiological form of Pol III HE was absent. Subsequently, single molecule studies were performed that concluded that Okazaki fragments contained gaps in rolling circle reactions reconstituted *in vitro* unless the triple polymerase form of Pol III HE was used (22). No correlation was made between these properties and the properties of the physiological replicase. Thus, although these studies show that a three-τ, three-polymerase form of Pol III HE can be constructed *in vitro*, they provide no evidence that the authentic Pol III HE in cells contains an enzyme of this composition.

More recently, an *in vivo* experiment was performed using the elegant technology provided by slimfield fluorescence spectroscopy (23). Using fluorescent fusions with chromosomally encoded replication proteins, it was concluded that there were 3.1 ± 1.1 Pol III α subunits and 3.1 ± 0.8 τs per replication fork. The authors, perhaps led by the findings presented in the preceding paragraph, concluded that the authentic cellular replisome was a trimeric polymerase containing three Pol III αs and three τs. A related study (24) came to nearly the same stoichiometric conclusion, but also showed a histogram of the number of molecules per fork that showed a broad distribution ranging from 1–8 Pol III αs per replication fork with a mean of 2.8 ± 0.6 (Supplementary Figure S17 in (24)). Clearly there cannot be more than three polymerases in the replicase. The histogram suggests that there may be more than one Pol III HE at the replication fork in some cases, increasing the number of molecules per fork and leading to a false interpretation that the mean molecules per fork represents the number of subunits in a single replicase. Consistent with this interpretation is the finding that if a mutant, which cannot frameshift and expresses only the τ form of DnaX, replaces the natural chromosomal copy of *dnaX*, the mean number of τs per replication fork increases by *ca.* 30%, *exactly* the result that would be expected if the physiological composition of the $DnaX_{cx}$ was $\tau_2\gamma\delta\delta'\chi\psi$ (23).

This controversy led us to design an approach that could unambiguously determine whether γ is a legitimate component of Pol III HE and test whether γ is an authentic translation product versus a proteolysis product of τ. To accomplish this, we purified Pol III* (all components of Pol III HE except β) from a strain with both a chromosomal *dnaX* that expressed only τ and a plasmid that expressed physiological levels of γ appended to a C-terminal tag that is biotinylated *in vivo*. The presence of γ containing this tag (γ-C_{tag}) could only be achieved by direct assembly of γ into Pol III HE *in vivo*. Proteolyzed τ would not contain the tag.

MATERIALS AND METHODS

Plasmids strains and manipulations

The expression plasmid pPRD-C (Table 1) contains a multiple cloning site downstream of the IPTG inducible promoter pA1 (25), a sequence encoding a four glycine linker, followed by a contiguous hexa-His tag sequence and a biotinylation sequence. This plasmid was constructed by replacing the sequence spanning the SpeI to KpnI sites of pDRK-C 5′ ACTAGTGGTGGTGGTGGTCTGGTTCC GCGTGGTTCCCACCACCACCACCTG CACCACA T CCTGGACGCTCAGAAAATGGTTTGGAACCAC TGCCGTTGATGAGGT ACC with 5′ ACTAG TG GTGGTGGTGGTCACCACCACCACCACCACGGT CTGAACGACATCTTCGAGGCTCAG AAAATCGA ATGGCACGAATGA TGAGGTACC.

The expression plasmid pA1-γ-C_{tag} was constructed by digesting pA1-CΔ213τ with SpeI and KpnI. The plasmid pA1-CΔ213τ encodes a C-terminally tagged *dnaX* corresponding to full-length γ cloned into the pDRK-C vector. The 98 bp SpeI/KpnI fragment in pA1-CΔ213τ was replaced with the 83 bp SpeI/KpnI fragment from pPRD-C generating pA1-γ-C_{tag}. The expressed gene product from the pA1-γ-C_{tag} plasmid, γ-C_{tag}, is a γ protein that is tagged at the C-terminus with a hexa-His and biotinylation sequence.

The strain PDEC105 was constructed by P1 transduction of the *dnaX*γ^-(Cam^R) from RRL330 (26) into MGC1030 bearing the plasmid pA1-γ-C_{tag} (27). Chloramphenicol-(30 μg/ml) and carbenicillin- (100 μg/ml) resistant transductants were subjected to two rounds of colony purification on LB plates (+chloramphenicol (30 μg/ml), carbenicillin (100 μg/ml), 5 mM sodium citrate) to ensure removal of residual P1 phage and separation of non-transduced bacteria. Genomic DNA was prepared from transductants (28) and polymerase chain reaction (PCR) was performed using oligonucleotide 166: 5′ TCAGACGCTGTTGATTG-GTC and 167: 5′ TCCAGCGATACGCTTCTTTT. PCR fragments amplified from genomic DNA (448 bp) bearing *dnaX*γ^- contained a diagnostic EarI restriction site which generates two fragments (301 and 147 bp) upon restriction digestion.

To generate PDEC106, PDEC105 was grown in the absence of carbenicillin overnight. Cells were plated onto LB + chloramphenicol (30 μg/ml). Individual colonies were picked in duplicate onto LB plates bearing either chloramphenicol (30 μg/ml) alone or LB plates containing chloramphenicol (30 μg/ml) and carbenicillin (100 μg/ml). Colonies that failed to grow in the presence of carbenicillin indicated that the strain had lost the pA1-γ-C_{tag} plasmid (2/100 screened).

Strains used in UV-sensitivity assays, mutagenic break repair (MBR) assays, transductional recombination and F′-copy-number studies that carry the I-SceI endonuclease gene and cutsite (‘DSB’) and the isogenic ‘No DSB′

Table 1. *Escherichia coli* strains and plasmids used in this work

Plasmid/Strain	Relevant genotype	Source	Lab strain #
pDRK-C	Expression plasmid 6-HIS (non-contiguous) tag and biotinylation sequence	(25)	
pPRD-C	Expression plasmid 6-HIS tag (contiguous) biotinylation sequence	This study	
pA1-CΔ213τ	IPTG inducible promoter, C-terminally tagged γ protein (6-HIS non-contiguous, biotinylation tag old)	(8)	
pA1-γ-C_{tag}	IPTG inducible promoter, C-terminally tagged γ protein (6-HIS contiguous, biotinylation tag new)	This study	
FC40	Δ(*lac-proB*)$_{XIII}$*ara thi* Rif^R [F' *proAB*$^+$ *lacI33-lacZ*]	(60)	
RRL330	*dna*Xγ$^-$ (Cam^R) F$^-$ λ$^-$ *thr*1 *leu*B6 *his*G4 Δ(*gpt-pro*A)62 *arg*E3 *lac*Y1 *gal*K2 *ara*C14 *xyl*A5 *mtl*1 *rps*L31(*str*R) *thi*1	(23)	2220
MGC1020	*mcr*A *mcr*B λ$^-$ IN(*rrn*D-*rrn*E)1 *lex*A3 Δ(*uvr*D):: $K_m{}^R$ *malE::TN10*	(19)	882
MGC1030	*mcr*A *mcr*B λ$^-$ IN(*rrn*D-*rrn*E)1 *lex*A3 Δ(*uvr*D)::$T_c{}^R$ Δ(*omp*T)::$K_m{}^R$	(19)	1422
PDEC105	MCG1030 *dna*Xγ$^-$ (Cam^R) + pA1-γ-C_{tag}	This study	2225
PDEC106	MCG1030 *dna*Xγ$^-$ (Cam^R)	This study	2226
SMR4562	Independent construction of FC40	(40)	
SMR4610	FC40 *recA*::Tn*10*dCam	(61)	
SMR6263	MG1655 *leu*:Tn*10*	(30)	
SMR6276	SMR4562 Δ*araBAD567* Δ*att*λ::P_{BAD}I-*Sce*I	(30)	
SMR6280	SMR4562 Δ*araBAD567* Δ*att*λ::P_{BAD}I-*Sce*I [F' *mhpA32*::miniTn7Kan(I-*Sce*I site)]	(30)	
SMR10865	Δ*zie3913.1*::*tetRtetA+1*FRT Δ*zie3920.5*::3ChiKanISceIsite	(35)	
SMR10866	Δ*att*λ::P_{BAD}I-*Sce*I Δ*zie3913.1*::*tetRtetA+1*FRT Δ*zie3920.5*::3ChiKanISceIsite	(35)	
SMR15956	SMR4562 *dnaX*γ$^-$(Cam^R)	SMR4562 x P1(RRL330)	
SMR22371	Δ*araBAD*567 Δ*zie3913.1*::*tetRtetA+1*FRT Δ*zie3920.5*::3ChiKanISceIsite *dnaX*γ$^-$(Cam^R)	SMR10865 x P1(SMR15956)	
SMR22372	Δ*araBAD*567 Δ*att*λ::P_{BAD}I-*Sce*I Δ*zie3913.1*::*tetRtetA+1*FRT Δ*zie3920.5*::3ChiKanISceIsite *dnaX*γ$^-$(Cam^R) –isolate 1	SMR10866 x P1(SMR15956)	
SMR22373	Δ*araBAD*567 Δ*att*λ::P_{BAD}I-*Sce*I Δ*zie3913.1*::*tetRtetA+1*FRT Δ*zie3920.5*::3ChiKanISceIsite *dnaX*γ$^-$(Cam^R) –independent isolate 2	SMR10866 x P1(SMR15956)	
SMR22376	SMR6276 *dnaX*γ$^-$(Cam^R)	SMR6276 x P1(SMR15956)	
SMR22379	SMR6280 *dnaX*γ$^-$(Cam^R) –isolate 1	SMR6280 x P1(SMR15956)	
SMR22380	SMR6280 *dnaX*γ$^-$(Cam^R) –independent isolate 2	SMR6280 x P1(SMR15956)	
SMR22381	SMR6280 *dnaX*γ$^-$(Cam^R) –independent isolate 3	SMR6280 x P1(SMR15956)	
SMR22382	SMR6280 *dnaX*γ$^-$(Cam^R) –independent isolate 4	SMR6280 x P1(SMR15956)	

strains that carry either the I-SceI cutsite but no I-SceI-enzyme expression cassette (e.g. SMR22371), or the enzyme but no cutsite (e.g. SMR22376), with or without *dnaX*γ$^-$, were constructed by P1 trandsduction (29) with selection in medium with added 0.2% D-fucose or 0.1% glucose, to repress I-*Sce*I cutting during strain construction per (30) and with or without 25μg/ml chloramphenicol to select the *cat* cassette (23) immediately downstream of *dnaX*γ$^-$ stop codon.

Buffers

The buffers used were: buffer A, 50 mM Tris–HCl (pH 7.5), 10% (v/v) sucrose; buffer I, 50 mM imidazole (pH 6.8), 20% (v/v glycerol), 5 mM dithiothreitol (DTT); S300 buffer, 50 mM HEPES (pH 7.5), 100 mM NaCl, 10% (v/v) glycerol, 0.25 mM ethylenediaminetetraacetic acid (EDTA), 0.01% NP40, 5 mM DTT; TRIS buffered saline (TBS), 20 mM TRIS (pH 7.4), 500 mM NaCl.

Media, cell growth and expression

Strains MGC1030, PDEC105 and PDEC106 were grown in 200 l F medium (14 g/l yeast extract, 8 g/l tryptone, 12 g/l K_2HPO_4, 1.2 g/l KH_2PO_4) + 1% glucose at 37°C. To maintain the plasmid (pA1-γ-C_{tag}) in PDEC105, media contained 50 μg/ml ampicillin. Expression of γ−C_{tag} was not induced with isopropyl–β-D-thio-galactopyranoside.

When strain PDEC105 reached OD_{600} = 0.8, additional ampicillin (50 μg/ml) was added. Cells were harvested when the OD_{600} was between 3–4. MGC1030 cells were harvested in a Sharples continuous flow centrifuge and re-suspended in 50% (v/v) buffer A. Cells were flash frozen in liquid nitrogen and stored at −80°C. For strains PDEC105 and PDEC106, cells were harvested and stored as cell paste at −80°C. M9 minimal medium is per (29) and contained 10μg/ml thiamine (vitamin B1), 0.1% glycerol, often with 0.2% D-fucose or 0.1% glucose to repress P_{BAD}I-SceI transcription, with or without 50μg/ml proline. Luria-Bertani-Herskowitz (LBH) medium is 1% tryptone, 0.5% NaCl, 0.5% yeast extract, 2 μg/ml thymine, pH 7 and for plates is solidified with 1.5% agar.

Purification of Pol III*

Preparation of cell lysates and ammonium sulfate precipitation. All procedures were performed at 4°C unless otherwise stated. A total of 500 g of cell paste were lysed as described (31) with the following exceptions: cells were lysed in the presence of 3 mg of lysozyme/g cells, 5 mM benzamidine and 1 mM phenylmethanesulfonyl fluoride, the solution was adjusted to pH 8.3 with 2 M Tris base solution and EDTA was added to a final concentration of 5 mM. To allow PDEC105 and PDEC106 cells to be amenable to digestion by lysozyme, they were resuspended in 2 l (final volume) 50 mM Tris (pH 8.3), 20 mM EGTA and incubated at 4°C for 2 h (32). Cells were collected by centrifugation (22 000 × *g*, 30 min), resuspended in buffer A and flash frozen in liquid N_2 and stored at −80°C until ready for lysis. Cells were thawed in a 37°C water bath, maintaining the temperature below 14°C (40–45 min)(33). The cell lysate was centrifuged (22 000 × *g* for 2 h) to remove debris preparing Fraction I.

Ammonium sulfate precipitation was carried out as described in (31). The procedure results in near quantitative precipitation of Pol III*, and removes contaminants by backwashing with decreasing concentrations of ammonium sulfate. The final pellet was flash frozen in liquid nitrogen and stored frozen at −80°C as Fraction II.

SP-sepharose. One-half of Fraction II was suspended in 20 ml of buffer I, dialyzed against 4 l buffer I for 3 h and diluted (final volume 60 ml) to a conductivity of 30 mM NaCl prior to loading a 20 ml SP-sepharose Fast Flow (GElifesciences.com, 1.5 × 10 cm) column pre-equilibrated with buffer I + 20 mM NaCl. The column was run at a flow rate of 0.5 ml/min. After loading, the column was washed with two column volumes of buffer I + 20 mM NaCl. Proteins were eluted with a 10 column volume gradient of buffer I + 20 mM NaCl to buffer I + 300 mM NaCl. Fractions that contained at least 50% of the activity of the peak fraction were pooled and precipitated by adjusting to 70% ammonium sulfate (0.436 g ammonium sulfate added to each ml of solution, 22 000 × *g*, 45 min) and stored at 4°C as Fraction III.

Q-sepharose. Fraction III was suspended in 6 ml buffer I, dialyzed against 4 l of buffer I for 3 h, and then diluted to a conductivity of <30 mM NaCl (ca. 25 ml) prior to loading a 4 ml Q-sepharose HP column (GElifesciences.com, 0.6 × 15 cm) pre-equilibrated with buffer I + 20 mM NaCl. The column was run at a flow rate of 0.25 ml/min. After loading, the column was washed with 10 column volumes of buffer I + 20 mM NaCl. Proteins were eluted with a 10 column volume gradient of buffer I + 20 mM NaCl to buffer I + 350 mM NaCl. Fractions that contained at least 50% of the activity of the peak fraction were pooled and precipitated with ammonium sulfate (0.436 g ammonium sulfate added to each ml of solution, 22 000 × *g*, 45 min) and stored at 4°C as Fraction IV.

Sephacryl S300. Fraction IV was suspended in a final volume of 200 μl of S300 buffer. Fraction IV was applied to a 20 ml S-300 HR (GElifesciences.com, 1 × 30 cm) column. The column was developed in S300 buffer at a flow rate of 0.2 ml/min and 0.4 ml fractions were collected. Fractions were not pooled at this point. However, calculations for Fraction V were made based on if we had combined fractions that contained a least 50% of the activity of the peak fraction. Individual fractions were flash frozen in liquid nitrogen and stored at −80°C.

Assays

Protein concentrations were determined by the method of Bradford using the Coomassie Plus Bradford reagent (Pierce) according to manufacturer's instructions using bovine serum albumin as a standard. Protein concentrations were measured in the linear region of the protein standard curve.

Pol III HE reconstitution assays were performed as described (34) in the presence of β_2 (150 fmol), DnaG primase (1 pmol), SSB_4 (15 pmol) in a total volume of 25 μl. Reactions were initiated by adding Pol III* last. One unit definition Pol III* activity is 1 pmol of total nucleotide incorporated into acid insoluble DNA per min.

SDS-PAGE gels and immunoblots

Purified protein fractions were denatured in sodium dodecyl sulphate (SDS) sample buffer and subjected to electrophoresis on 4–20% gels (NuSEP, Bio-Rad). For visualization of protein, gels were stained with a Coomassie GelCode Blue Stain Reagent (Thermo scientific) according to manufacturer's instructions. For biotin blot and immunoblot analysis, proteins were transferred to nitrocellulose at 100 V (ca. 250 mA) for 1 h. The membrane was blocked overnight with TBS containing 5% nonfat biotin-free milk (LabScientific.com). Blots were washed one time in TBS 10 min, followed by two washes in TBS-Tween 0.1% for 10 min each, and one more wash in TBS for 10 min.

Biotin blots were incubated with streptavidin-alkaline phosphatase conjugate (Thermo scientific, 1:1000 dilution) in TBS-milk 5% for 2 h. For quantification, blots of γ_4 standards were scanned and a standard curve prepared (DnaX assembles as a tetramer in the absence of δ and δ' (14)). The signal from the Pol III* 'unknowns' was compared to the standard curve and the quantity of biotinylated γ present was calculated.

For immunoblots with DnaX antibody, membranes were incubated with a mouse monoclonal antibody (AB251)

against DnaX that recognizes both τ and γ protein (1:1000 dilution in TBS-milk 5% overnight). Wash steps were repeated and then blots were incubated in goat anti-mouse alkaline phosphatase conjugated antibody (Bio-Rad) at a 1:1000 dilution for 2 h. For detection, wash step steps were repeated and the blots were visualized using alkaline phosphatase conjugate substrate kit (Bio-Rad) that contains nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates.

Preparation of biotinylated γ_4 standard and determination of percent biotinylation

Purified γ_4 was prepared from strain PDEC105. Cells (4 l) were induced with 1 mM isopropyl–β-D-thiogalactopyranoside (3 h), harvested by centrifugation, protein extracts prepared, and protein subjected to Ni^{2+}-nitrilotriacetic acid column chromatography as described (34). Biotinylated γ_4 was purified by binding and elution from a monomeric avidin column (Promega Avidin Softlink resin, 2 ml bed volume). Pooled material from the Ni^{++} NTA column (*ca.* 6 mg, 9 ml) was loaded directly onto the pre-equilibrated avidin column and re-circulated five times at a flow rate of three column volumes per h. The column was washed 10 column volumes of equilibration buffer (0.1 M sodium phosphate pH 7.4, 0.15 M NaCl, 5 mM DTT, 10% glycerol) and eluted with 10 column volumes of elution buffer (equilibration buffer + 2 mM D-biotin). The first column volume was eluted at a flow rate of two column volumes per h. For the second column volume, the flow was stopped and the column was allowed to incubate overnight. In the morning, the remaining nine column volumes were eluted at a flow rate of 1 column volume per h. Any remaining bound material was eluted from the column by the addition of 4 column volumes of regeneration buffer (0.1 M glycine (pH 2.8)) at 3 column volumes per h.

The percent biotinylation of γ_4 was determined using a combination probability formula for the number of combinations $_nC_r = n!/r!(n-r)!$ where $_nC_r$ represents the number of combination of n things (here, set arbitrarily at 100 so results are given as percentages) with a set size r that is equivalent to the oligomerization state of the protein (4 for γ_4).

The fraction (F) of γ_4 that is completely non-biotinylated is given by: $F = {}_{(n-x)}C_r/{}_nC_r = (100-x)!/4!((100-x)-4)!/100!/4!(100-4)!$ where x = the overall percent biotinylation of the γ_4 sample applied to the avidin column. We experimentally determined the fraction of γ_4 that was completely unbiotinylated by quantification of the column flow through (0.62). Solving for x indicated that 11% of the monomers in the applied γ_4 were biotinylated.

The percentage (P_e) biotinylation of monomers within the eluted biotinylated γ_4 fraction is given by $P_e = F_L/F_B \times 100$ where F_L is the overall fraction of γ monomers biotinylated in the fraction applied to the column (0.11) and F_B is the fraction of γ_4 bound to the column (0.38). Thus, the percentage of biotinylated monomers in the eluted fraction was 29%.

To quantify biotinylated γ-C_{tag} within Pol III*, standard curves were constructed using purified γ_4 that had been determined to be 29% biotinylated. Densitometric scans were processed using ImageJ software (imageJ.NIH.gov) and one plotted density unit was 10 000 arbitrary integrated density units using that program.

UV-sensitivity semi-quantitative assays

Cells were grown to saturation overnight in LBH with 0.1% glucose at 37°C with shaking. The saturated cultures were diluted 10-fold and 3 μl spots were arrayed onto LBH 0.1% glucose agar plates, and the plates successively irradiated to the final UV doses shown, using a Stratagene UV Stratalinker®. Irradiated plates were covered with aluminum foil to prevent photo-reactivation repair of UV-induced DNA damage and incubated at 37°C for ca. 6–7 h to assay density of cfu survival.

Chromosomal mutagenic-break-repair assays

For each experiment, 3–6 independent cultures were prepared per strain to be assayed. For each culture, a single colony from M9 0.1% glucose proline vitamin B1 (B1) plates was inoculated into 5 ml M9 proline 0.1% glucose B1 liquid, then incubated with shaking at 37°C for 8–10 h. Each was diluted 1:100 into 5 ml of the same medium, and grown to prolonged stationary phase by shaking at 37° for 3 days. This modification of the protocol of (35), skips a subsequent, non-essential dilution. The cultures were then assayed for total viable and tetracycline-resistant (Tet^R) cfu by serial dilution (for total cfu) and plating onto LBH 0.1% glucose solid medium and LBH 0.1% glucose 10 μg/ml tetracycline plates, respectively. Colonies were scored after ~18 h at 37°C, and the titers of Tet^R mutant and total cfu used to determine the frequencies of Tet^R mutant cfu per total cfu.

Quantitative phage P1-mediated transduction assays for homologous-recombination proficiency

Aliquots of saturated LBH 0.1% glucose cultures of recipient strains were diluted, then plated onto LBH 0.1% glucose agar plates to determine viable cfu titers. Separate aliquots of the undiluted saturated cultures were mixed with suspensions of phage P1 grown on donor strain SMR6263, which carries the tetracycline-resistance (Tet^R) marker *leu*::Tn*10* (30) and transduction reactions performed per (29) with the following modifications. First, all media contained 0.1% glucose to repress P_{BAD}I-SceI. Second, these transduction reactions were conducted with at least 10× more cells than P1 phage, titers determined per (29), such that P1 particles are limiting for transduction. This allows transduction efficiency to be expressed as Tet^R transductants per P1 plaque-forming unit (pfu) e.g. (36). After a 20 min incubation of phage with cells, cells were pelleted and resuspended in LBH 0.1% glucose with 20 mM sodium citrate, then incubated at 37°C shaking for an additional 60 min before being spread onto LBH solid medium with 0.1% glucose, 3.33 μg/ml tetracycline and 20 mM sodium citrate. Plates were then incubated overnight at 37°C, and Tet^R transductant colonies scored the following day.

Flow cytometric assay for counting numbers of *E. coli* chromosomes

Two to three independent cultures per strains were grown to prolonged stationary phase exactly as for chromosomal MBR assays. A total of 0.5 ml of each culture was mixed with an equal volume of 5% paraformaldehyde, incubated at room temperature for 15 min, and then on ice for additional 45 min to fix the cells. The fixed cells were washed twice with 1 ml M9 liquid, resuspended in 1 ml M9 containing 50 μg/ml DAPI and incubated for 1 h in darkness before flow cytometric analyses. Cells were analyzed by a BD LSR Fortessa^{TM} at the Cytometry and Cell Sorting core at Baylor College of Medicine. To obtain a DAPI-positive 'gate,' the cultures were compared with preparations of strain SMR10866 that were fixed but not DAPI stained. A DAPI-positive gate was defined as the range of DAPI fluorescence intensity exceeded by $\leq 5 \times 10^{-5}$ of unstained SMR10866 cells. Approximately 10^5 DAPI-positive cells were analyzed per culture. Synchronized bisector gates (FloJo, LLC) were used to divide cells with two or more chromosomes from cells with fewer than two, to isolate the peak with higher DAPI fluorescence intensity across histograms.

RESULTS

We previously demonstrated that proteolysis of the τ subunit of Pol III HE post-lysis could be prevented by avoiding prolonged dialysis at early stages of purifications or by performing purifications from strains in which the structural gene for the offending protease (OmpT) had been inactivated (19). Purification of τ-complex ($\tau_3\delta\delta'\chi\psi$) expressed from an artificial operon in an *ompT* strain showed that there are no other proteases that degrade τ to a protein near the size of γ (19). Thus, we performed our standard Pol III HE purification (31) with appropriate precautions from a strain containing wild-type *ompT* and a strain that was *ompT*$^-$. Both purifications yielded equivalent amounts of γ relative to τ and α (Figure 1), suggesting γ is a legitimate component of Pol III HE.

To unambiguously demonstrate that γ assembles into Pol III HE when expressed at normally wild-type protein levels, we exchanged the chromosomal copy of *dnaX* with one that contains a mutation that ablates frameshifting but does not change the amino acid sequence of τ. γ containing a C-terminal tag that is biotinylated *in vivo* was expressed from a plasmid (Figure 2). Using the natural biotin carrier protein as a standard, we were able to determine that the plasmid expresses ~75 molecules/cell (Figure 3). This level approximates the 180 molecules total DnaX/cell estimated previously (23). Two additional control strains were also employed—one wild-type relative to *dnaX* (MGC1030) and the other containing the chromosomal *dnaX* mutation that expresses only τ (PDEC106; Figure 2).

A four-step purification procedure was adapted to purify Pol III* from all three strains, resulting in a purification of at least 2000-fold (Tables 2–4). The elution profile from the final purification step (S300 gel filtration) from each of the purifications is shown along with an SDS gel of the peak fractions and a biotin-blot indicating any biotinylated protein present in the final purified material (Figures 4–6).

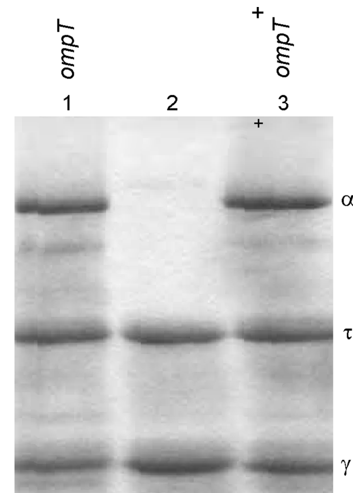

Figure 1. Pol III holoenzyme purified from wild-type and *ompT* strains contains equivalent levels of γ subunit. Poll III HE was purified from MGC1020 (wild-type *ompT*$^+$) (lane 1) and MGC1030 (*ompT*$^-$) (lane3). Purified γ and τ standards were run in Lane 2. Approximately 8 μg of protein was loaded to each lane.

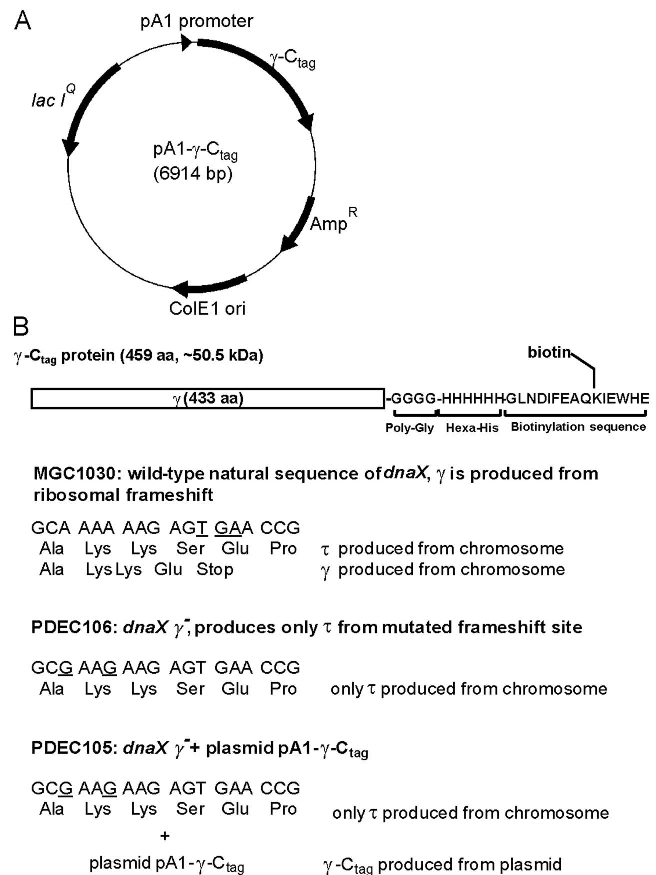

Figure 2. Plasmid and strains used to purify Pol III* complexes. (**A**) Map of plasmid pA1-γ-C$_{tag}$. (**B**) Three different strains were used to purify Pol III* complexes: wild-type (MGC1030 *ompT, dnaX*$^+$) produces Pol III* complexes that contain γ and τ; PDEC106 produces Pol III* complexes that contain only τ; and PDEC105 produces Pol III* complexes that contain both τ (from chromosomal *dnaX*γ-) and γ-C$_{tag}$ (from pA1-γ-C$_{tag}$ plasmid).

Table 2. Purification of Pol III* complex from PDEC105 (*dnaX*γ^- + pA1-γ-C_{tag})

Fraction	Total protein (mg)	Total units	Specific activity units
i. Lysate	10 200	3.4×10^6	330
ii. Ammonium sulfate	350	3.9×10^6	16 000
iii. SP-Sepharose	15	2.5×10^6	160 000
iv. Q-Sepharose HP	2.1	8.3×10^5	400 000
v. S-300	0.62	4.8×10^5	770 000

Table 3. Purification of Pol III* complex from PDEC106 (*dnaX*γ^-)

Fraction	Total protein (mg)	Total units	Specific activity units
i. Lysate	12 000	3.1×10^6	270
ii. Ammonium sulfate	220	2.2×10^6	10 000
iii. SP-Sepharose	14	3.3×10^5	25 000
iv. Q-Sepharose HP	0.8	1.3×10^5	150 000
v. S-300	0.1	2.6×10^4	440 000

Table 4. Purification of Pol III* complex from MGC1030 (wild-type *dnaX*)

Fraction	Total protein (mg)	Total units	Specific activity units
i. Lysate	19 000	6.2×10^6	340
ii. Ammonium sulfate	210	2.7×10^6	20 000
iii. SP-Sepharose	8.2	1.0×10^6	130 000
iv. Q-Sepharose HP	0.81	6.3×10^6	770 000
v. S-300	0.15	3.0×10^5	2 000 000

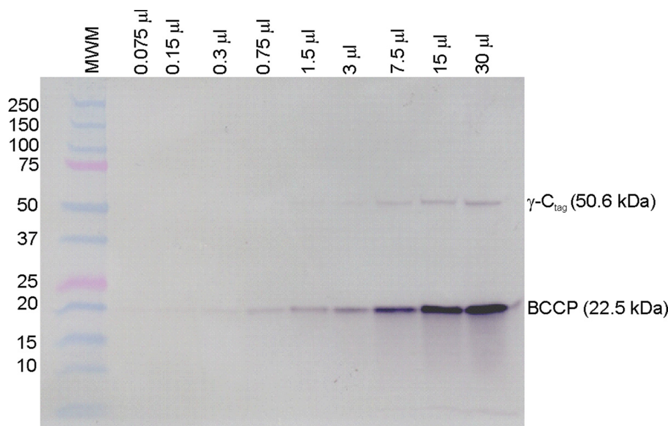

Figure 3. pA1-γ-C_{tag} expresses 75 molecules of γ-C_{tag} per cell. Strain PDEC105 was grown to OD_{600} = 0.5 in the absence of IPTG induction. Cells were harvested, boiled in SDS sample buffer, and the indicated amount of sample was loaded onto the lanes of a 4–20% SDS-PAGE gel. The gel was transferred to nitrocellulose and probed for biotinylated proteins using an alkaline phosphatase-conjugated streptavidin that detects both the native biotin carboxyl carrier protein (22.5 kDa) (BCCP = 3000 molecules/cell (62)) and γ-C_{tag} (50.5 kDa). The signal of γ-C_{tag} (at 30 µl) was equivalent to the signal of that of BCCP at 0.75 µl, indicating the level of expression of γ-C_{tag} is 40-fold lower than BCCP or 75 molecules/cell.

In the key experiment, bands corresponding to plasmid-expressed γ-C_{tag} and chromosomally expressed τ are visible on the gel (Figure 4B), paralleling the intensity of the Pol III α band and its activity (Figure 4A). There was an additional band just slightly larger than γ-C_{tag} that eluted with the activity peak, but with peak intensity slightly to the right (circled in Figure 4B). We confirmed the identity of the γ and identified the band of slower mobility as inosine monophosphate dehydrogenase (Supplementary Figure S2 and associated text). The biotin blot (Figure 4C) indicates that γ is biotinylated and thus not a proteolysis product of τ.

In control experiments, we observed that only τ (no γ) is expressed from PDEC105, which has a chromosomal mutation that only expresses τ (Figure 5B). This was confirmed by an immunoblot (Supplementary Figure S1). From MGC1030 (wild-type chromosomal *dnaX*), we observed both τ and γ associated with Pol III α and activity, as expected (Figure 5A,B). A contaminant of slightly higher mobility than γ elutes slightly later in the column profile. Thus, the parallel elution of τ and γ with the activity can be seen most easily by comparison of the anti-DnaX western blot in Supplementary Figure S1 panel A with the activity profile (Figure 6A). As expected, no biotinylated γ is present in either purification profile (Figures 5C and 6C).

The preceding experiments demonstrate that γ-C_{tag} is assembled into Pol III HE when expressed in *trans* from a plasmid. We wanted to quantify γ-C_{tag} to determine whether it was present in sufficient quantity to be a stoichiometric component of Pol III HE. To accomplish this, we purified overproduced, biotin-tagged γ to provide a quantitative standard. Using statistical methods described under 'Materials and Methods' section, we determined that the biotinylation level of γ_4 tetramers eluting in the presence of biotin from a soft-link avidin column was 29%. Using this material to generate a standard curve (Figure 7), we determined that there were 0.023 ± 0.005 ng γ-$C_{tag/}$ ng Pol III*. Using a stoichiometry of $(\alpha\epsilon\theta)_2\tau_2\gamma\delta\delta'\chi\psi$, γ should constitute 7.6% of the mass of Pol III*. It is our experience that the biotin tag we use is incompletely biotinylated, so that needed to be taken into account for stoichiometric calculations. In this study (see 'Materials and Methods' section)

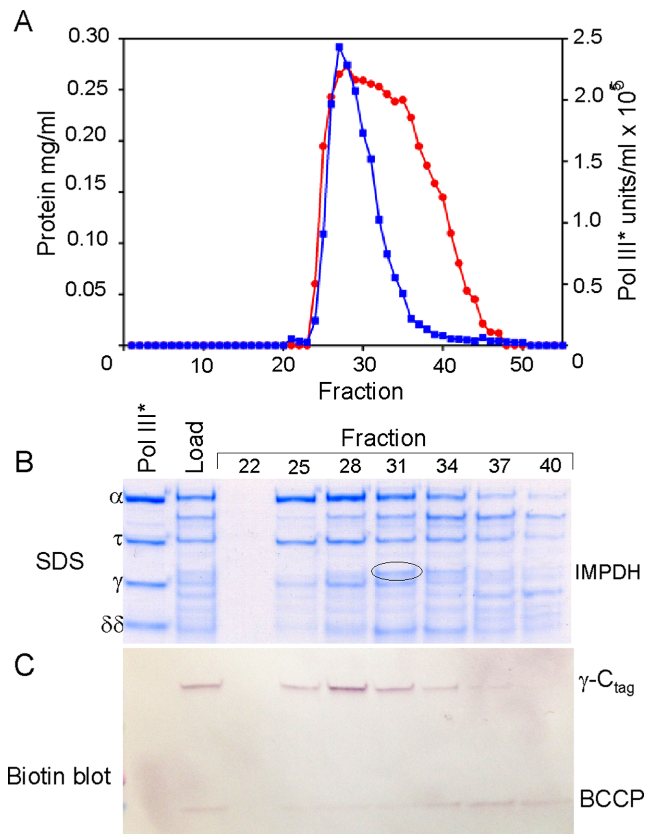

Figure 4. Purification of Pol III* from PDEC105 (*dnaX*γ^- + pA1-γ-C_{tag}). (**A**) S300 gel filtration column profile used to generate Fr V. The two plots indicate protein concentration (red circles) and Pol III* activity (blue squares). (**B**) Coomassie stained 4–20% SDS-PAGE gel of fractions from S-300 column. (**C**) Biotin blot of protein fractions from S300 column using streptavidin-alkaline phosphatase as probe (1:1000 dilution, 16 min exposure). BCCP (biotin carboxyl carrier protein, 22.5 kDa), τ (71 kDa), γ-C_{tag} (50.5 kDa).

overproduced γ was 11% biotinylated overall. Recently purified α and β was determined to be 31 and 17% biotinylated, respectively (unpublished observation). A gel of our purified Fr V Pol III* from PDEC105 shows that γ-C_{tag} can be resolved into two bands when detected by an anti-DnaX immunoblot (Supplementary Figure S3). The upper band is biotinylated (as indicated by comparison to a biotin blot) and constitutes 31 ± 7% of the total γ protein. Using this correction, 7.4 ± 2.3% of the mass of the Pol III* purified from PDEC105 is made up of γ, in close agreement with one γ per Pol III*. It follows that, since only τ binds Pol III, this limits the Pol III stoichiometry to two.

We show that γ functions in cells by demonstrating that cells that produce only τ and no γ from a mutant *dnaX* gene are abnormal in at least two respects. First, resistance to ultraviolet light (UV) requires multiple DNA-repair and DNA-damage-tolerance mechanisms, some of which use DNA Pols I (37), II and III (38) and V (39). We find that cells that produce only τ and no γ are sensitive to UV (Figure 8A). These data demonstrate that γ plays some functional role in *E. coli*.

Second, cells that produce only τ and no γ show reduced mutagenic break repair (MBR), a DNA Pol IV-dependent

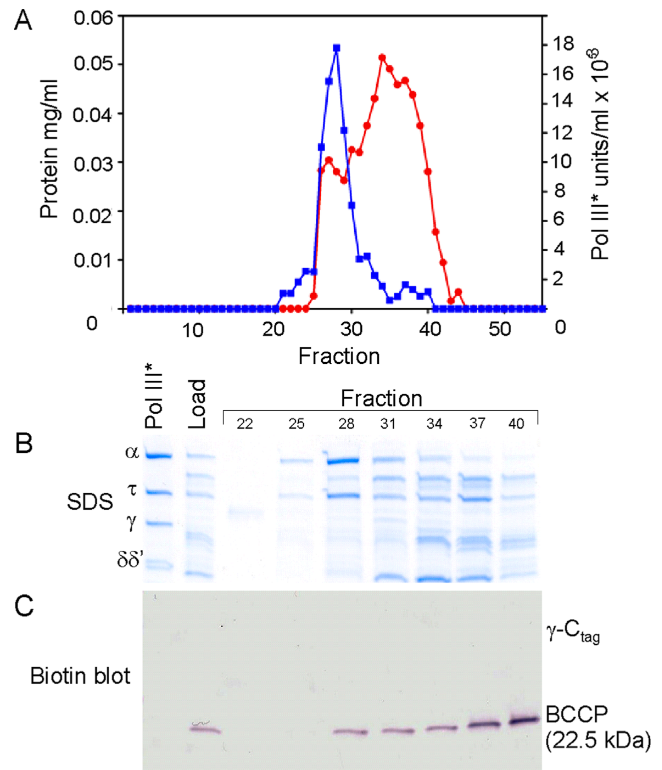

Figure 5. Purification of Pol III* from PDEC106 (*dnaX*γ^-). See legend to Figure 4.

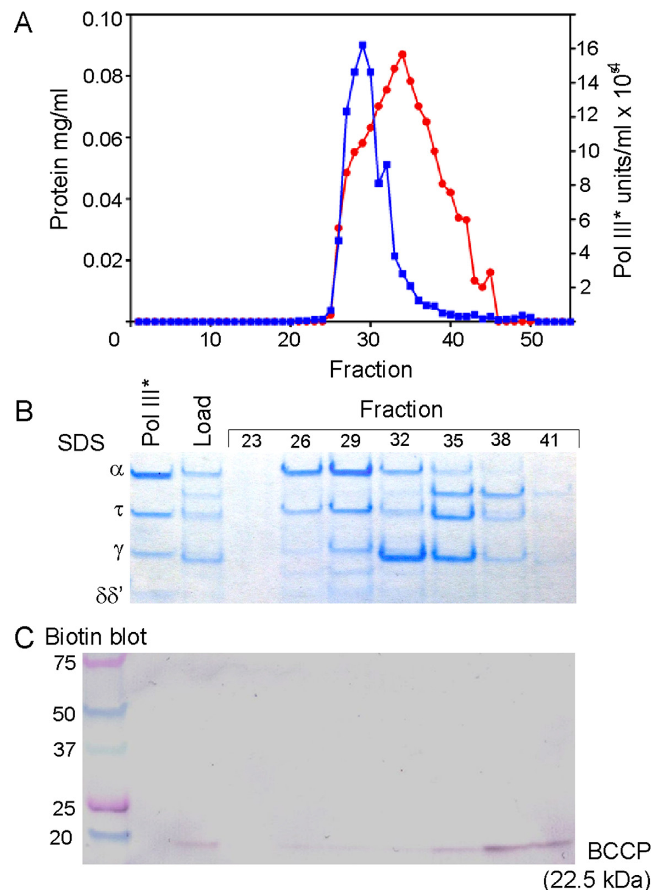

Figure 6. Purification of Pol III* from MGC1030 (WT). See legend to Figure 4.

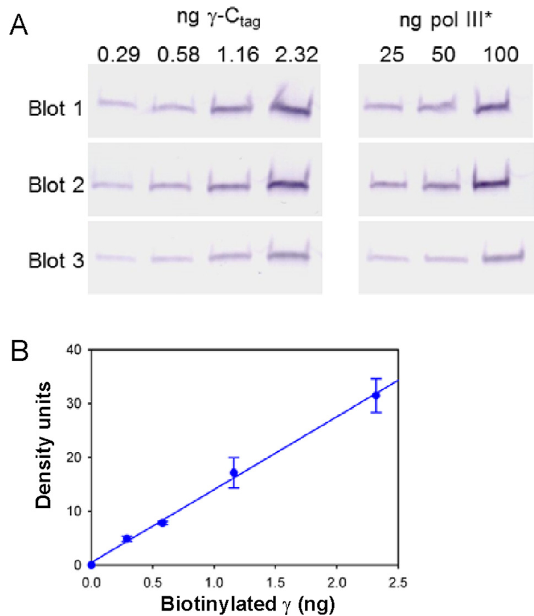

Figure 7. Purified Pol III* from PDEC105 (*dnaX*γ^- + pA1-γ-C_{tag}) contains *ca.* 1 γ subunit per complex. (**A**) Quantitative biotin blots were run in triplicate with purified γ-C_{tag} (29% biotinylated) to generate a standard curve. The peak Pol III* fraction V (#28) was run in triplicate. (**B**) The densities from Pol III* bands were analyzed and compared with standard curves. We calculated 0.023 ng of biotinylated γ monomers per ng of Pol III*.

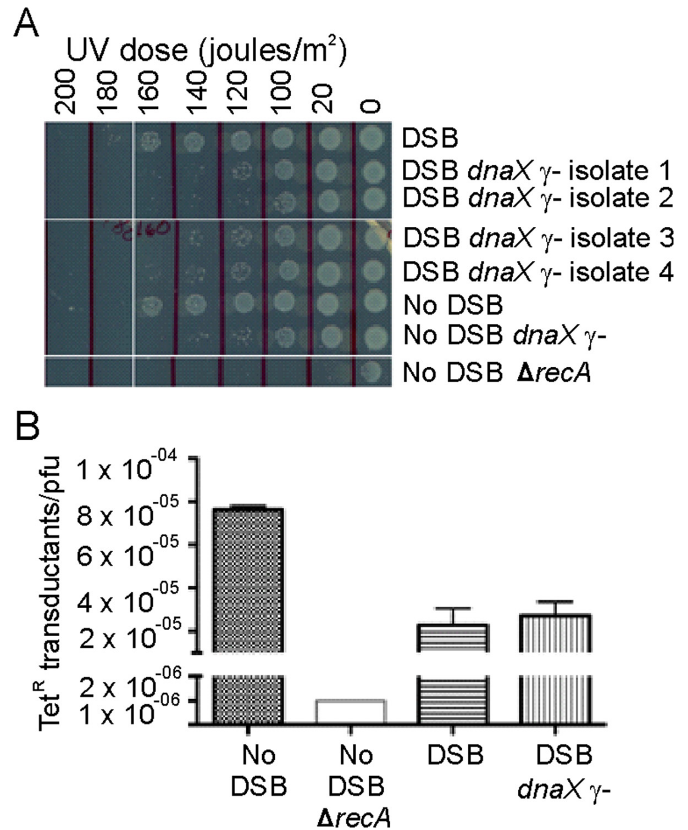

Figure 8. Mutants strains that produce only τ and no γ clamp-loader subunits are sensitive to ultraviolet light but homologous-recombination proficient. (**A**) Representative data show density of cfu after exposure to UV at the doses indicated. The chromosomal P_{BAD}I-*Sce*I gene was repressed by addition of glucose, so that both I-SceI-cleavage-proficient ('DSB') and -deficient ('No DSB') isogenic strain sets are assayed without additional DNA damage from I-SceI cleavage. Strains, from top to bottom: SMR6280; SMR22379; SMR22380; SMR22381; SMR22382; SMR6276; SMR22376. 'No DSB' has the P_{BAD}I-*Sce*I gene but no cutsite. (**B**) τ-only mutant cells are proficient at transductional recombination. Tetracycline-sensitive τ-only recipient strains were transduced with phage P1 grown on donor strain SMR6263, which carries a tetracycline-resistance (Tet^R)-conferring Tn*10*. All stages of these experiments were conducted in 0.1% glucose to block I-SceI cleavage in 'DSB' strains. Recipient strains: No DSB, SMR4562; No DSB *ΔrecA*, SMR4610; DSB, SMR6280; DSB *dnaX*γ^-,SMR22379, SMR22380, SMR22381 and SMR22382.

process (30,35,40). Repair of double-strand breaks (DSBs) in *E. coli* occurs by homologous recombination (HR), Pol III-dependently (41). DSB repair is high-fidelity and relatively non-mutagenic in cells that are not stressed, but switches to a mutagenic mode, using Pols IV, V and II, when cells have both the SOS DNA-damage response and the general stress response activated (30,35,42–43). The general stress response somehow licenses the use of Pol IV and other alternative DNA polymerases in DSB repair, with Pol IV being the major contributor (43).

We used the MBR assay of Shee *et al.* (35) in which cells carry a regulatable I-SceI endonuclease and chromosomal I-SceI cutsite near a *tet* frameshift mutation-reporter gene (Figure 9A). The cells are starved in stationary phase, rescued from starvation then assayed for tetracycline resistant (Tet^R) mutant cfu, which result from −1 bp deletions in a run of 5Gs (35). We find that cells that produce only τ display a significant 44% reduction in DSB-dependent Tet^R mutant frequency relative to isogenic strains with wild-type *dnaX* ($P = 0.001$, Student's *t*-test, two-tailed, two-sample equal variance indicated by *, (Figure 9B).

The following data indicate that reduced MBR results from reduced mutagenesis associated with DSB repair, not reduced ability to repair DSBs. DSB repair could be impaired either by lack of sister chromosomes with which to repair a DSB or by failure of HR at DSB ends. Using flow cytometry to count DAPI-stained chromosomes, we found that both the starved τ-only mutants and cells with wild-type *dnaX* have more than one (usually two) chromosome copies in about half to three-fourth of cells (Figure 9C representative data, Table 5, two experiments). The fraction with two chromosomes varies in experiments done on different days, but is similar between wild-type and τ-only cells on a given day (Figure 9C, Table 5), and also similar to the value of 45% of stationary-phase *E. coli* with two chromosomes reported previously (44). The τ-only mutant cells are also proficient in HR of linear DNA, seen in assays of transductional recombination of linear DNA with the bacterial chromosome (Figure 8B). They recombine like wild-type and unlike HR-defective *recA* cells (Figure 8B). These data imply that neither MBR-deficiency nor UV-sensitivity of τ-only mutants results from defective DSB repair. We conclude that although DSBs are repaired in τ-only mutant cells, the Pol IV-dependent mutagenesis associated with DSB repair is reduced.

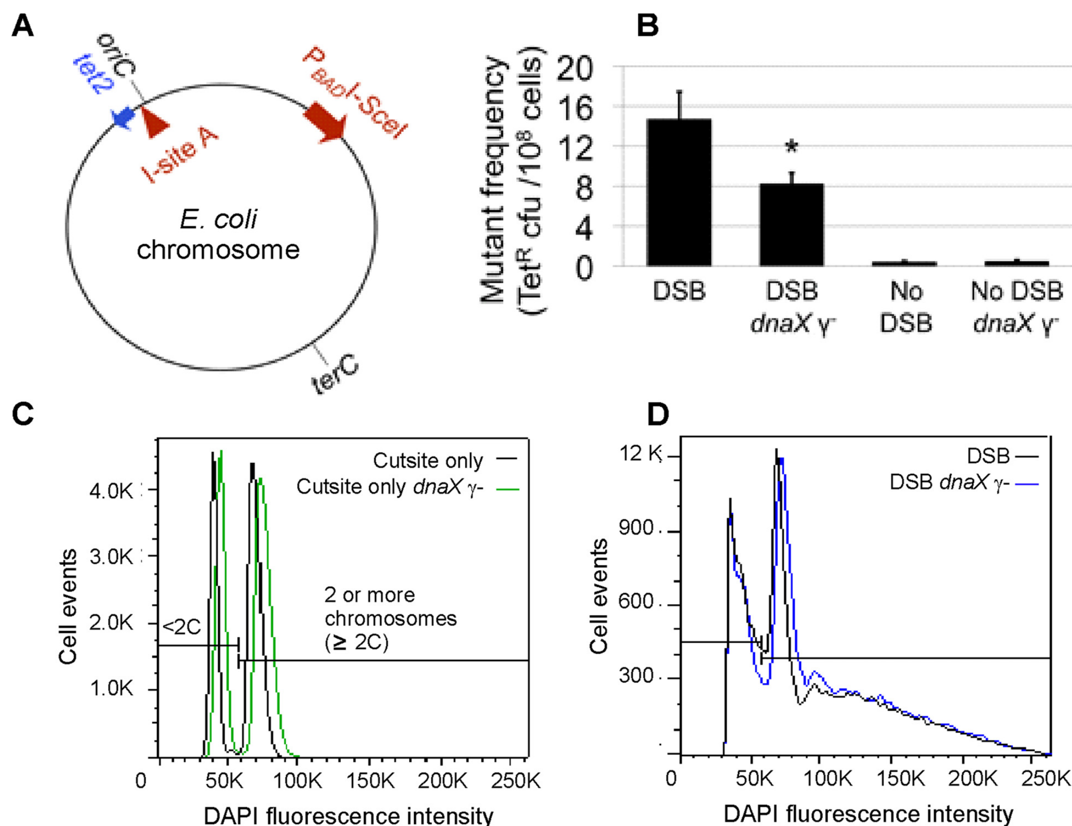

Figure 9. Clamp-loader subunit γ promotes mutagenic double-strand break repair. (**A**) Mutagenic-break-repair (MBR) assay of Shee *etal*. (35). Cells with a chomosomal regulatable I-*Sce*I endonuclease gene, I-*Sce*I cutsite (I-site A) and the *tet2* frameshift-mutation-reporter gene nearby are starved in stationary phase, and revert their *tet2* gene by MBR. MBR produces tetracycline-resistant (Tet^R) mutant cfu, assayed after rescue from starvation, dependently on Pol IV, HR-DSB-repair proteins, RpoS and SOS responses (30,35). Strains: DSB, SMR10866; DSB *dnaX*γ^- SMR22372, SMR22373; No DSB SMR10865, No DSB *dnaX*γ^- SMR22371. (**B**) The γ clamp-loader subunit promotes MBR. Cells that produce only τ and no γ display a significant (44%) reduction in DSB-dependent Tet^R mutant frequencies relative to isogenic wild-type *dnaX* strains (*$P = 0.001$, Student's *t*-test, two-tailed, two-sample equal variance). Mean ± SEM, seven independent experiments for 1–6 independent strain constructions of each genotype. I-*Sce*I induced Tet^R mutant frequency 33 ± 3-fold (seven experiments ± SEM) in the wild-type DSB strain compared with the 'No DSB' control, (I-*Sce*I cutsite but no endonuclease gene). The absence of γ did not significantly decrease Tet^R mutant frequency in the cells that were not cleaved with I-SceI (No DSB strains). (**C**) The τ-only mutation does not reduce chromosome copy number and prevent repair thereby. Representative flow-cytometry histograms of numbers of chromosome copies ('Materials and Methods' section) in wild-type and τ-only cells prepared for the MBR experiments, either without (**C**) or with (**D**) I-SceI cleavage. Data from this and a second experiment are shown in Table 5. Peaks corresponding to one and two chromosome copies are less well resolved in DSB strains than in cutsite-only strains, indicating probable break-induced replication/repair synthesis. Strains: cutsite only, SMR10865; *dnaX*γ^- cutsite only, SMR22371; DSB, SMR10866; DSB *dnaX*γ^-, SMR22372, SMR22371.

Table 5. Chromosome copy number is unaffected by τ-only mutant *dnaX* (flow cytometry data)

Strain	Relevant genotype	Exp	% of cells with <2 chromosomes[1]	WT/*dnaX*γ^-
SMR10865	Cutsite only	1	43%	1.0
		2	22%	0.92
SMR22371	Cutsite only *dnaX*γ^-	1	41%	
		2	24%	
SMR10866	DSB	1	28%	0.97
		2	31%	1.3
SMR22372	DSB *dnaX*γ^-	1	29%	
		2	23%	

[1]Mean of 2–3 independent cultures per experiment (Exp) determined by flow-cytometric analysis (see Materials and Methods) per Figure 9C.

The data show that living cells use γ and are abnormal without it, displaying defects in resistance to UV light (Figure 8) and Pol IV-dependent mutagenesis (Figure 9).

DISCUSSION

Prior to several recent proposals that the *E. coli* replicase is a trimeric polymerase *in vivo* (21–24), considerable information was available that suggested otherwise. Evidence included: (i) all preparations of chromosomally encoded Pol III HE contained γ (31,45), (ii) proteolysis of τ to generate a γ-sized protein could be prevented by avoiding prolonged dialysis of crude extracts or by purifying enzyme from an *ompT* mutant (19), (iii) purified recombinant DnaX complex expressed from a plasmid that expressed only the τ form of DnaX did not contain γ, yet $DnaX_{cx}$ purified from a nearly identical plasmid that contained wild-type *dnaX* expressed both γ and τ that assembled into the same complex (19), (iv) immunoprecipitation of Pol III HE using a monoclonal antibody against the unique C-terminus of τ also precipitated γ, indicating they are part of the same assembly (17) and (v) γ occupies a unique location in the $DnaX_{cx}$ (20). It would be unusual indeed for artifactual proteolysis to cleave a DnaX subunit at only one of three locations within the pentameric ring created by $DnaX_3\delta\delta'$. None of these observations were considered in the papers proposing that the Pol III HE was a trimeric polymerase lacking γ.

We recognize that the observation that τ is not cleaved to γ when overproduced might have been due to a limiting level of protease. So, we first examined the composition of Pol III HE purified from an *ompT* strain and one with wild-type *ompT*, but purified without prolonged dialysis, preventing OmpT-mediated proteolysis. We observed the same levels of γ in both preparations, suggesting that it is an authentic subunit of Pol III HE.

To provide a more definitive test, we constructed an *ompT* strain that carried a chromosomal *dnaX* gene that was mutated at the frameshifting site so that it only expressed τ. We introduced γ into this strain through a plasmid that expressed γ with a C-terminal tag that is biotinylated *in vivo*. If γ-C_{tag} assembled into otherwise chromosomally encoded Pol III* *in vivo* in *trans*, that would prove it to be a legitimate component of Pol III HE and not a proteolysis product. Pol III* purified ~2000-fold from this strain indeed contained biotinylated γ. Quantitative immunoblots indicated one molecule of γ per Pol III HE assembly. A control strain that only expressed τ yielded Pol III* that lacked γ.

These results definitively prove that authentic γ, not a proteolysis product of τ, assembles into Pol III HE. Since only the τ form of DnaX binds Pol III α, this limits the Pol III stoichiometry in Pol III HE to two. The two initial proposals (21,22) were from enzyme reconstituted *in vitro* in the absence of γ. No properties of this assembled enzyme were correlated with native Pol III HE, so there is no logical link that supports the trimeric Pol III HE proposal for the enzyme expressed in cells.

Our data show that the average stoichiometry of γ within Pol III HE is one and do not directly preclude a low level of enzyme with a τ_3 stoichiometry. Indeed, if the assembly of the DnaX-complex was stoichastic, one would expect a distribution that included all possible stoichiometries of DnaX within Pol III HE. γ occupies a unique location in the $DnaX_{cx}$ (20) within the Pol III HE, an observation that is incompatible with a stoichastic assembly mechanism. A model for near exclusive assembly of Pol III HE with a $\tau_2\gamma$ stoichiometry that is partially supported by kinetic and binding data has been proposed (1).

The data presented by fluorescence microscopy studies of replication forks can be reconciled with our findings. Those studies showed 3 ± 1 molecules of Pol III α and τ per replication fork on average (23,24). A histogram showed a broad distribution of the number of α subunits per replication fork, ranging as high as eight with a mean of three (24). We interpret this as indicating that more than one Pol III HE can be associated with a replication fork. One would be the replicative polymerase with a leading and lagging Pol III coupled by two τ subunits in the $DnaX_{cx}$. A second Pol III HE, sometimes present, could be involved in mismatch repair or processing of gaps left in the lagging strand. It could also be present in a non-functioning association of its τ subunit with subunits of the $DnaB_6$ replicative helicase that are not bound to the replicative Pol III HE (8). The finding that replacement of chromosomal *dnaX* with a mutant that only expresses τ increased the mean stoichiometry of τ at the replication fork by one-third (23) provides strong evidence, in our view, that the natural stoichiometry of DnaX is $\tau_2\gamma$ within the $DnaX_{cx}$. Other issues pertaining to experimental design and interpretation of the fluorescence microscopy experiments have been discussed in a review article (section 5.1 of (1)).

Having established that γ is a component of Pol III HE that functions in cells, the question remains of why it exists and why it is a component of the Pol III HE. Our understanding of γ has been largely defined by what it does not do rather than what its unique contributions are to the properties of the bacterial replicase. γ contains the ATPase activity required to load a β_2 sliding clamp on DNA, but it lacks the α binding domain required to chaperone Pol III onto a nascently loaded clamp, allowing the reaction to proceed at a physiologically relevant rate. γ also lacks the DnaB binding domain of τ that is required to accelerate the helicase and couple Pol III HE to the replication fork. Since γ lacks the Pol III α binding domain, it is also unable to couple leading and lagging strand replication.

γ is found in organisms as diverse as *E. coli*, *Caulobacter crescentus,* and *Thermus thermophilus* and is synthesized by three distinct mechanisms (2–4,46–47). Such widespread conservation is testimony to the utility of γ. Yet, the function of γ has been a topic that has not been carefully investigated. *E. coli* mutants that apparently do not frameshift and produce only the τ form of DnaX are viable (48), but the fitness of these mutants has not been examined.

In this study, we demonstrated for the first time that *E. coli* cells that lack γ display abnormal phenotypes, demonstrating that not only do these cells make γ, but they require its presence for normal cellular function. We found that cells making only τ and no γ are sensitive to UV light and impaired in Pol IV-dependent mutagenesis associated with DSB repair.

The presence of error-prone polymerases has been shown to be important for generation of mutations for evolution and fitness (49–53). In *E. coli*, the error-prone polymerases

Pol IV and Pol V are thought to transiently exchange with Pol III by interaction with an unoccupied site on the β_2 (49,54–56). Yet, the interaction of such polymerases with β_2 is weak (μM) (57,58). It would appear that the presence of an 'extra' Pol III at the replication fork, created by association with τ in τ_3-containing replicases, would likely outcompete the other polymerases (59). Thus, the presence of γ within Pol II HE may be essential to allow exogenous polymerases to compete at the replication fork, not by an active role, but by avoiding the presence of a third copy of Pol III at the replication fork that would otherwise outcompete them because of its high local concentration. This hypothesis could explain why replication appears to proceed at a near normal rate in τ-only *dnaX* mutants even though expression of γ and τ from *dnaX* is widespread in bacteria. Our observation that *E. coli* mutants that express only τ exhibit decreased levels of Pol IV-dependent mutagenesis are consistent with this hypothesis. *Caulobacter* strains that cannot express γ are inviable (47). However, *Caulobacter* that express γ in *trans*, perhaps altering the balance of the γ and τ forms, are more sensitive to the DNA damaging agent mitomycin C and display decreased levels of UV-induced mutagenesis (47).

Thus, we have demonstrated that γ is a legitimate component of the *E. coli* replicase. Expression from *dnaX* to produce γ is essential for normal cellular function in DNA metabolic roles ranging from sensitivity to a DNA damaging agent and decreased mutagenesis by an error-prone polymerase. These observations open exciting avenues for future investigation of the functions of the short alternative product of *dnaX* in bacteria and the influence of relative levels of the two *dnaX* proteins on important cellular processes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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