

Replisome-mediated Translesion Synthesis by a Cellular Replicase*

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Running Title: DNA Polymerase III Holoenzyme Trans-lesion Synthesis

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*This work was supported, in whole or in part, by National Institutes of Health Grant GM34557 (to K. J. M.) and Cancer Center Core Support Grant NCI P30CA008748 (to MSKCC).

This article contains supplemental Table S1 and Fig. S1

Keywords: DNA, DNA enzyme, DNA polymerase, DNA replication, DNA repair, DNA damage

Genome integrity relies on the ability of the replisome to navigate ubiquitous DNA damage during DNA replication. The *Escherichia coli* replisome transiently stalls at leading-strand template lesions and can either reinitiate replication downstream of the lesion or recruit specialized DNA polymerases that can bypass the lesion *via* translesion synthesis. Previous results had suggested that the *E. coli* replicase might play a role in lesion bypass, but this possibility has not been tested in reconstituted DNA replication systems. We report here that the DNA polymerase III holoenzyme in a stalled *E. coli* replisome can directly bypass a single cyclobutane pyrimidine dimer or abasic site by translesion synthesis in the absence of specialized translesion synthesis polymerases. Bypass efficiency was proportional to deoxynucleotide concentrations equivalent to those found *in vivo* and was dependent on the frequency of primer synthesis downstream of the lesion. Translesion synthesis came at the expense of lesion-skipping replication restart. Replication of a cyclobutane pyrimidine dimer was accurate, whereas replication of an abasic site resulted in mainly -1 frameshifts. Lesion bypass was accompanied by an increase in base substitution frequency for the base preceding the lesion. These findings suggest that DNA damage at the replication fork can be

replicated directly by the replisome without the need to activate error-prone pathways.

DNA damage is ubiquitous and cells have evolved ways to ensure that DNA lesions that escape repair can be tolerated during DNA replication. A prevailing model is that high-fidelity replicative DNA polymerases are unable to replicate through lesions in the DNA, leading to replisome stalling and arrested DNA replication followed by release of the replication block by a number of DNA damage tolerance pathways (1,2). These pathways include (a) the recruitment of specialized DNA polymerases that can switch with the stalled replicative polymerase, bypass the DNA damage by translesion synthesis (TLS)², and then allow the replicative polymerase to resume replication; (b) restart pathways in which the replisome “skips” the damaged DNA and reinitiates replication downstream of the lesion at a newly synthesized primer, leaving a gap behind as a substrate for TLS or recombination-dependent template switch mechanisms; and (c) fork reversal followed by template switching. While these pathways allow replication to proceed, they slow replication progression by the recruitment of proteins and remodeling of the replication fork and can come with a mutagenic cost because of error-prone TLS polymerases and the potential for fork breakage. Because most replication forks will encounter DNA damage (1) and replication is not

completely blocked by DNA damage (3), replisomes may be able to bypass DNA damage by a more direct mechanism. Indeed, replicative polymerases have been suggested to be directly involved in TLS *in vivo* (4-9).

E. coli has five DNA polymerases (DNA Pol) (10), three of which, Pol II, Pol IV [DinB (11)], and Pol V [UmuD'₂UmuC (12)], are considered specialized TLS polymerases (13). The DNA Polymerase III Holoenzyme (Pol III HE; the abbreviation Pol III will be used to refer directly to the core polymerase) is the replicative polymerase in *E. coli* that catalyzes both leading- and lagging-strand synthesis. It is composed of two polymerase cores [α (the catalytic polymerase subunit), ϵ (the 3'→5' exonuclease), and θ] that are held together in the HE particle by a dimer of the τ subunit of the DnaX complex ($\tau_2\gamma\delta\delta'\chi\psi$) that also includes the processivity clamp-loading activity, and two dimers of the processivity clamp, β (14). Like other replicative DNA polymerases, its constrained active site and 3'→5' exonuclease proofreading activity enable it to copy DNA with high fidelity but with limited tolerance for template lesions (13). While it is believed that the Pol III HE is unable to bypass UV lesions such as a cyclobutane pyrimidine dimer (CPD), *dnaE*, encoding the catalytic α subunit of the HE (15,16), has been shown to be required for UV mutagenesis (17) in *E. coli* (4,5) and there is evidence that proofreading-deficient Pol III can bypass a CPD *in vivo* in the absence of TLS polymerases (6), implicating the Pol III HE in a more direct role in lesion bypass. Moreover, the balance between proofreading and elongation by Pol III depends on dNTP concentration (18,19) and it has been shown that increasing the dNTP concentration results in increased TLS in the absence of known TLS polymerases in *E. coli* (7), suggesting that Pol III may perform TLS directly. DNA damage induces upregulation of dNTP levels in bacteria (7) and yeast (20) and elevated dNTP levels reduces the fidelity of DNA replication in bacteria (7), yeast (20), and mammalian cells (21).

Using a reconstituted DNA replication system with which we can observe the collision of the *E. coli* replisome with leading-strand template damage, we demonstrate that when incorporated in a replisome, the Pol III HE can bypass a single site-specific CPD or the abasic site analog

tetrahydrofuran (THF) by direct TLS. Bypass is proportional to dNTP concentration, occurs at concentrations found under normal growth *in vivo*, is very efficient at dNTP concentrations found in SOS-induced cells, and competes directly with lesion-skipping replication restart (22). These observations may define a fourth potential pathway of DNA damage tolerance by the replisome and suggest that UV-mutagenesis at the replication fork in *E. coli* may be a result of Pol III HE-catalyzed replication errors and not the action of the damage-inducible DNA Polymerase V.

Results

Pol III Can Directly Bypass CPD and THF Lesions In a Replisome Context—To investigate the ability of the *E. coli* replisome to bypass a single lesion in the leading-strand template we utilized a replication system (22) supported by a 10.4-kbp plasmid containing the *E. coli* origin of replication, *oriC*, and either a CPD or THF lesion located on the leading-strand template 6.9 kb downstream of *oriC* (Fig. 1A). In this system *oriC*-dependent replication is initiated using DnaA, DnaB, DnaC, DnaG, HU, SSB, Pol III HE (from a TLS pol⁺ strain), and DNA gyrase. The counterclockwise-moving fork is blocked by Tus at two *terB* sites located approximately 400 bp away from *oriC*, resulting in an unidirectional replication system in which the clockwise-moving replisome, which will encounter the DNA damage on the leading-strand template, can be monitored. Replication reactions were carried out in the presence of radiolabeled nucleotides for 8 min and quenched by the addition of AMP-PNP and ddNTPs, followed by digestion with EcoRI and PvuI, and analysis using both denaturing- and native agarose gel electrophoresis. Replication on five different templates was compared: undamaged, CPD-containing, and three THF-containing templates. The latter three templates varied by the position of the THF lesion in the template strand and are denoted as THF1, THF2, and THF3 (Fig. 1A). Fig. 1A also shows the products expected after digestion with EcoRI and PvuI for complete replication on undamaged DNA, lesion skipping replication restart, uncoupling of leading- and lagging-strand synthesis, and lesion bypass (22,23).

Replication of an undamaged DNA template resulted in mainly full-length, leading-

strand products of 9.6 kb as well as shorter lagging-strand products of 0.5 – 2.0 kb, as demonstrated by denaturing gel analysis (Fig. 1B, lane 1). Because the nascent lagging strand, but not the leading strand, terminates 50 – 70 bp upstream of the *terB* site (24), EcoRI will not cut the lagging-strand sister duplex, resulting in the full-length, lagging-strand duplex products migrating more slowly than the full-length, leading-strand duplex products (9.6 kbp) after EcoRI-PvuI digestion (22). As demonstrated by native gel analysis, replication of an undamaged template results in equal proportions of full-length, leading- and lagging-strand sister duplexes, as well as some larger products resulting from incomplete EcoRI-PvuI digestion (Fig. 1C, lane 1).

Replication of a template containing a single CPD or THF under standard reaction conditions resulted in a 6.7 kb leading-strand fragment as a result of replisome stalling at the lesion, as well as a shorter 2.8 kb leading-strand fragment produced from replication restart downstream of the lesion (Fig. 1B, lanes 2, 4, 6, and 8). Notably, no intact full-length leading-strand products were produced, indicating complete stalling of the replisome under these reaction conditions. The full-length replication products resulting from restart can be observed by native gel analysis (Fig. 1C), showing that while there are no intact, full-length leading strands observed in the denaturing gel, there are equal proportions of full-length, leading- and lagging-strand sister duplex products resulting from DnaG-dependent restart of coupled replication downstream of the lesion (Fig. 1C, lanes 2, 4, 6, and 8). Stalled forks that were not restarted can be observed in the native gel migrating close to the 23 kbp marker together with uncut products (Fig. 1C). Broken stalled forks result in a 6.7 kbp duplex and represent a minor fraction of the products (Fig. 1C). Some template unwinding occurs after fork stalling because of continued helicase progression (25), but in this assay the products from such unwinding will not be cut by EcoRI and will therefore migrate as large Y-structures close to the stalled fork product.

In contrast to the standard reaction conditions, replication of a CPD-containing template in the presence of elevated concentrations of dNTPs [40 μ M dNTPs and an additional 750 μ M dATP and dCTP; this concentration was used

to maximize TLS, which is proportional to dNTP concentration (see Fig. 3)] resulted in intact, full-length leading-strand products, indicating direct lesion bypass by the replisome (Fig. 1B, compare lanes 2 and 3). As for the standard conditions, there were equal proportions of full-length leading- and lagging-strand products in addition to the stall product (Fig. 1C, compare lanes 2 and 3), indicating that the replisome remains coupled during the DNA damage bypass. Similar results were obtained for the THF1 template (Fig. 1B and 1C, compare lanes 4 and 5). The average fraction of full-length products resulting from direct bypass was 0.32 ± 0.07 ($n = 5$) for the CPD template and 0.15 ± 0.8 ($n = 5$) for the THF1 template (Fig. 1D). Elevated dNTP concentrations did not result in significant lesion bypass with the THF2 and THF3 templates (Fig. 1B, lanes 6-9 and Fig. 1D), possible reasons for this are considered in the Discussion.

Lesion Bypass is Stimulated by Elevated Concentrations of Specific Nucleotides—Because of the nucleotide dependence of the lesion bypass observed, we tested which nucleotides were required for the replisome to bypass the CPD and THF lesions. Replication of either the CPD or THF1 templates in the presence of increased concentrations (790 μ M) of dTTP, dCTP, or dGTP, did not result in lesion bypass (Fig. 2A, lanes 4-6, and 10-12); whereas elevated dATP concentration resulted in a small amount of bypass on both the CPD (5%) and THF1 (3%) templates (Fig. 2A, lanes 3 and 9). In contrast, increasing the concentration of both dATP and dCTP resulted in substantial bypass on both the CPD (40%) and THF1 (30%) templates (Fig. 2, lanes 7 and 13). To test whether lesion bypass was simply dependent on increased concentration of any dNTP rather than that of specific dNTPs, we performed replication reactions in the presence of elevated concentrations of combinations of two dNTPs. Whereas increased concentrations of dATP and dTTP (Fig 2B, lanes 2 and 6), as well as of double the concentration of dATP (Fig. 2B, lanes 3 and 7) resulted in a limited amount of bypass (8% and 13%, respectively for the CPD template; < 5% for the THF1 template), as was observed for dATP alone (Fig. 2A, lane 3), only increased concentrations of dATP and dCTP resulted in robust bypass (50% for the CPD template; 30% for the THF1 template) (Fig. 2B,

lanes 4 and 8). Indeed, the amount of lesion bypass is directly proportional to the concentration of dATP and dCTP (Fig. 3). It is important to note that Pol III HE-TLS will occur *in vitro* at concentrations of dNTPs found *in vivo* (see Discussion).

The Stalled Nascent Leading Strand Is the Precursor for Lesion Bypass—To demonstrate that it was indeed the replisome that stalled at either the CPD or THF lesion that progressed to full-length, lesion-bypass products, we employed a variation of the replication reaction that is synchronized and allows us to perform pulse-chase experiments (22). In this reaction, replication is initiated at *oriC* in the absence of any topoisomerase leading to the accumulation of positive supercoils that stall the clockwise-moving replisome about 1 kb from the origin. Replisomes are then released by rapid EcoRI digestion of the template at the same time as radiolabeled nucleotide is added. After 1 min, the labeled nucleotide is chased with an 100-fold excess of cold nucleotide together with additional dATP and dCTP and replication is allowed to continue for 6 min. In this reaction, replication of the CPD template under standard nucleotide conditions resulted in stall products that were converted to full-length duplexes over time *via* replication restart downstream of the lesion (Fig. 4A, native gel, lanes 1-3). Note that in this reaction, EcoRI cleavage occurs before the replisome reaches the *terB* site, thus the full-length, leading- and lagging-strand sister duplexes are of equal size. In the presence of increased concentrations of dATP and dCTP, replication of the CPD template resulted in stalled forks efficiently being chased into intact full-length products, as determined by denaturing gel analysis. After 6 min of replication in the presence of elevated nucleotide concentrations, 40% of the stalled replisomes had bypassed the CPD and 20% had bypassed the THF (Fig. 4A lanes 4-6 and Fig. 4B). Thus, stalled replisomes can directly bypass a CPD or THF in the presence of elevated dATP and dCTP by extending the leading-strand stall product directly across the lesion site.

Lesion Bypass Competes with Replication Restart Downstream—We have reported (25) that when the leading-strand polymerase stalls at a lesion in the leading-strand template, the lagging-strand polymerase and the helicase continue

downstream, albeit at a reduced rate, until primase synthesizes a new primer on the unwound leading-strand template. The leading-strand polymerase then cycles forward to this new primer, skipping over the lesion and coupled leading- and lagging-strand replication resumes. Lesion bypass, on the other hand, would seem to require increased residency of the leading-strand polymerase at the lesion and thus should be likely to compete with the lesion-skipping reaction. The gap formed in the nascent leading strand increases during the lesion-skipping reaction as an inverse function of the primase concentration, indicating that at lower concentrations of primase it takes longer for a target primer to be made off the leading-strand template. We reasoned that the efficiency of lesion bypass should be similarly inversely related to primase concentration. At lower concentrations of primase residency of the leading-strand polymerase at the lesion should increase as, then, should lesion bypass. We therefore examined the effect of primase concentration on the bypass reaction.

Replication of the CPD template in the absence of DnaG did not produce any products (Fig. 5A, lanes 1 and 8). As expected, when DnaG concentrations were decreased, the length of the Okazaki fragments increased (26) (Fig. 5A, lanes 2-7). Notably, when the primase concentration was decreased to below 40 nM, the replisome was able to bypass the CPD and produce the 9.6 kb full-length, leading-strand product under standard dNTP reaction conditions (Fig. 5A, lanes 2 and 3; Fig. 5B). To confirm that this observation was the effect of priming frequency, we utilized the variant DnaG-Q576A, which has a reduced affinity for the DnaB helicase and produces longer Okazaki fragments than wild type at identical concentrations because of less frequent priming on the lagging-strand template (27). Replication of the CPD template in the presence of DnaG-Q576A resulted in bypass products at higher concentrations of primase in comparison to wild type (Fig. 5A, compare lanes 9-14 to lanes 2-7). We conclude that at limited primase concentrations, the leading-strand polymerase is less likely to cycle forward to a new primer on the leading-strand template, thereby promoting lesion bypass. Thus, lesion bypass and lesion skipping by the replisome are competing pathways.

The Replisome Accurately Bypasses a CPD But Generates -1 Frameshifts When Bypassing a THF—To gain insight into the lesion bypass mechanism and determine the fidelity of bypass, we isolated the full-length, leading-strand duplexes resulting from lesion bypass and determined the sequence of the nascent leading strand. Strikingly, deep sequencing revealed that two adenine nucleotides were inserted opposite the CPD in over 98% of cases (Table 1), indicating mostly accurate bypass of the lesion. This observation is consistent with a mechanism by which Pol III bypasses a CPD of either direct insertion of a nucleotide opposite the damage site by TLS or utilization of the “A” rule (28). No significant replication errors were observed in the sequencing of products produced from replication of an undamaged template. For the THF lesion, a majority (52%) of products were -1 frameshifts (Table 1 and supplemental Fig. S1), indicating bypass *via* template misalignment. An adenine nucleotide was inserted opposite the THF 46% of the time, consistent with the “A-rule” of DNA polymerases (28).

For both the CPD and THF lesions, bypass was associated with slight, but significant, increases in base substitution frequency at the lesion with significantly greater increases for the base directly preceding the damage (Table 1 and Fig. S1), consistent with the stalled replisome being stuck in a futile cycle of nucleotide insertion and exonuclease activity.

The Pol III HE Alone Cannot Bypass Either a CPD or THF Lesion Using Oligonucleotide Primer-template Substrates—Previous reports had indicated that the Pol III HE itself had little or no capability of bypassing template lesions (23,29,30), as usually assumed for replicative DNA polymerases, suggesting that the bypass we have observed was a special property of the replisome. To confirm that the Pol III HE was unable on its own to bypass template lesions, we performed primer extension assays using two different oligonucleotide primer-template (p/t) substrates: One comprised only of the primer and template to test the ability of Pol III HE to bypass a lesion directly, and another, forked substrate, on which the DnaB helicase could be loaded to test whether the interaction between the HE and DnaB was required for lesion bypass. On these substrates there are three nucleotides

between the 3' end of the primer and the template lesion. Using an undamaged p/t, Pol III HE was able to extend the primer to the end of the template (Fig. 6, lane 1), whereas efficient primer extension using the forked substrate required the presence of DnaB helicase (Fig. 6, compare lanes 4 and 10). The presence of a THF or CPD template lesion resulted in Pol III stalling at the +3 position, right before the template lesion, even in the presence of DnaB (Fig. 6, lanes 2 and 3, 5 and 6, 8 and 9; and 11 and 12). These data indicate that both a CPD and a THF lesion represent a strong block to the polymerization activity of the Pol III HE, thus indicating that the observed bypass described in this report is likely a gain of function as a result of the incorporation of the HE into a replisome. We suspect that the failure of DnaB to stimulate bypass on these p/t's is likely because of the failure to successfully form a complex with the τ subunit of the HE in the short time available during replication of any particular template.

Discussion

Pathways of Replication Fork Reactivation—Collisions of replication forks with template damage are a major source of genomic instability (1,2). Whereas damage in the lagging-strand template is not usually a major problem—the lagging-strand polymerase cycles past the damage and initiates the synthesis of a new Okazaki fragment—damage in the leading-strand template will stall the leading-strand polymerase, requiring the action of other enzymatic pathways to ensure that replication can continue past the damage. These pathways of DNA damage tolerance fall into two broad categories: error-prone and error-free. Error-free pathways depend on homologous recombination to allow the fork to progress. Generally, after dissociation or removal of the replisome proteins, the stalled fork is remodeled by nascent strand regression, whereby the nascent leading- and lagging-strands pair with each other driving the replicated nascent duplex backward. This provides a template for direct correction of the error by, e.g., nucleotide excision repair, the fork is then restored and replication restart is then required. Remodeled forks can also serve as a template for strand switching events that, once the fork is restored, allow progression past the lesion. These pathways are error free. Error-prone pathways involve the recruitment of a

specialized lesion bypass polymerase that may switch with the stalled leading-strand polymerase, insert a base opposite the lesion, and then at some point further downstream synthesis can switch back to the replicase. TLS polymerases tend to be significantly more error-prone than cellular replicase. Another pathway involves lesion-skipping, where the stalled leading-strand polymerase can cycle forward downstream to a new primer made off the leading-strand template, allowing fork progression to resume. This pathway can be either error-prone or error-free depending on how the template damage left behind at a gap in the nascent leading-strand is repaired. Daughter-strand gap repair would be error-free, whereas TLS across the lesion followed by filling in of the gap would be error-prone. We have described in this report another fork reactivation pathway that is relatively error free, direct TLS bypass of template damage by a cellular replicase.

TLS By the Cellular Replicase—We report that the *E. coli* cellular replicase, the DNA Pol III HE can bypass leading-strand template lesions when it is in the context of the replisome. Bypass of a CPD lesion was by direct insertion of nucleotides opposite the lesion, whereas bypass of a THF abasic site analog was a mixture of direct insertion and -1 frameshifts. Given that THF bypass was much reduced when the lesion was in the middle or at the end of a run of three template thymidine residues, the preferred mode of THF bypass is likely template slipped mispairing (31). Pol III HE alone could not bypass either template lesion on short oligonucleotide primer-templates, whether in the presence or absence of the DnaB helicase, indicating that bypass was a special property conferred on the HE by virtue of being engaged in the replisome, catalyzing simultaneous leading- and lagging-strand synthesis while also physically engaged with the helicase.

X-ray crystal structures of TLS and cellular replicase polymerases has revealed a striking difference in the disposition of the active sites for polymerization. Cellular replicases tend to be tightly apportioned, with no “room” in the active site to accommodate either non-standard base conformations or rotation of template bases out of line with the template duplex, whereas the active sites of TLS polymerases are far “roomier,” able to tolerate such non-standard deviations

(32,33). The observations reported here suggest that such a view is somewhat parochial, the active site of the α subunit of the HE must be flexible enough to tolerate both a CPD template lesion and template slipped mis-pairing.

The Role of Elevated Concentrations of Deoxynucleoside Triphosphates—Pol III HE TLS bypass under conditions of saturating primase required nucleotide concentrations in excess of those typically used in our DNA replication systems *in vitro*. In standard polymerization assays, the K_M for dNTPs of the HE is in the low micromolar range, hence we’ve always considered our standard concentration of 40 μ M dNTPs to be more than adequate to sustain maximum DNA replication. The concentration of dNTPs in the cell is hard to pin down, there is significant variability in published reports, but, in general, reports indicate them to be in the range of 50 - 250 μ M, albeit they are individually unbalanced in concentration (34-36).

Our observations show, not surprisingly, a requirement for nucleotide concentration in excess of our standard conditions of the nucleotides that are inserted directly opposite the lesion and the next nucleotide called for downstream of the lesion. However, significant TLS is, in fact, observed at the presumed cellular concentrations. We suspect these observations reflect the fact that the catalytic efficiency of TLS and extension of the nucleotides opposite the lesion is less than the normal catalytic efficiency of polymerization. Thus, elevated concentrations of nucleotide facilitate the process by licensing the rate-limiting chemical step.

Mutator phenotypes have been attributed to DNA replication errors caused by altered dNTP pools (7). It has been suggested that increasing the concentration of dNTPs shifts Pol III from a proofreading mode to an elongation mode in which proofreading is diminished. Indeed, it has been shown that the base substitution rate is proportional to dNTP concentration (18) and that extension from a mismatch depends on concentration of the next correct dNTP (37). However, these observations are not sufficient to explain the data presented here because, in that case, TLS should be detectable using a primer-template oligonucleotide substrate. As shown in Fig. 6, the HE is completely blocked by a CPD or THF on an oligo substrate even in the presence of

750 μ M dNTPs. Furthermore, Pol III HE TLS was clearly observed at our standard nucleotide concentration when the concentration of Primase was reduced in the replication reaction (Fig. 5). Thus, whereas shifting the balance between proofreading and elongation may play some role in the observed Pol III HE TLS, it is unlikely to be the main driving force. Because we observed lesion bypass by Pol III only in a replisome context, our data indicate that TLS by Pol III requires a coupled replisome.

TLS and Lesion-skipping By the Replisome Are Inversely Related—The role of primase and priming frequency in TLS suggests a relationship between primase-dependent lesion skipping and TLS. It has been shown that lesion skipping and replication restart requires a downstream primer to be readily available. As the stalled leading-strand polymerase waits for a primer to become available on the leading-strand template downstream of the lesion, template unwinding and lagging-strand synthesis continue (25), generating a single-stranded DNA loop on the leading-strand template that is presumably an eventual effector for induction of the SOS response. Our data suggest that in the absence of a readily available downstream primer and under conditions where the HE can replicate past lesions, such as elevated dNTP concentration, TLS will occur rather than continued uncoupled replication. Thus, it is likely that the stalled leading-strand polymerase is engaged in a futile cycle of attempts to replicate past the lesion and degradation of the nascent DNA. The effect of the mutant DnaG is particularly telling in this respect. Here, rather than facilitating the licensing of the chemical step of polymerization across the lesion by increasing the nucleotide concentration, we increase the dwell time of the leading-strand polymerase at the lesion by increasing the time required before a new primer is made downstream on the leading-strand template. Where the break even point is between TLS and lesion skipping is unknown, but in either case the net result is suppression of SOS induction under unstressed conditions when there are relatively few lesions in the chromosomal DNA.

The nature of the gain in function for TLS by the HE in the replisome is unclear. It is possible that it relates to increased stability of the leading-strand polymerase when it is stalled at the

lesion. We have previously demonstrated that an interaction between the χ subunit of the HE and SSB stabilizes the leading-strand polymerase in the replisome (38). Thus, SSB on the gap generated in the nascent leading-strand sister downstream of the stalled leading-strand polymerase by continued unwinding may actually contribute to TLS by stabilizing the leading-strand polymerase. Alternatively, stretching of the physical connection between α and τ as continued unwinding occurs may, in turn, lead to a distortion of the leading-strand template that favors TLS. This latter possibility was favored by Sun et al. (9) in their analysis of TLS by the bacteriophage T7 DNA polymerase.

Replisome-mediated Pol III HE TLS and SOS Mutagenesis—Witkin discovered that spontaneous mutagenesis increased when *E. coli* cells wild type for *lexA* (then *exrA*) were exposed to UV irradiation, a phenomenon that has become known as SOS- or UV-induced mutagenesis (17). Not long afterward Bridges *et al.* (5) reported that a function of *dnaE* was required for UV-induced mutagenesis. The discovery that *umuC* and *umuD* were required for UV-mutagenesis (39,40) led to the hypothesis that these gene products were somehow modifying the Pol III HE to allow TLS, perhaps by inactivating the proofreading subunit ϵ or directly modifying the α subunit (41,42). However, the subsequent discovery that UmuC was a DNA polymerase and that when complexed with RecA-mediated, auto-digested UmuD formed the TLS polymerase DNA Polymerase V, UmuD₂'UmuC (12), directed attention away from the Pol III HE as the possible agent of mutagenesis.

UmuDC also play a role in chromosomal untargeted mutagenesis (UTM) (43). This transient increase in mutations in chromosomal genes after SOS induction is characterized by an increase in transversions (43-45), a mutation signature not thought to be a result of the HE acting alone. And, indeed, Livneh's group has demonstrated that Pol V generates mainly transversions (46). However, Moses' group (4) isolated *pcbA1*, a mutation that suppressed the temperature-sensitivity of the *dnaE346* allele. These strains were dependent on *polA* for viability and did not show any UV mutagenesis at the non-permissive temperature. UV mutagenesis could be restored by providing a plasmid *in trans*

expressing *dnaE*. In addition, Woodgate's group demonstrated that a proofreading-deficient Pol III HE could perform TLS in an otherwise TLS-deficient strain (6). Thus the role of the Pol III HE in UV mutagenesis remains unclear.

Our studies show that the Pol III HE is a very efficient TLS polymerase under conditions similar to those found under SOS induction where it has been reported that nucleotide concentrations increase by a factor of 2-4 (7). Furthermore, we show that the frequency of base substitutions increases during Pol III HE-catalyzed TLS at the template position prior to the CPD and in the first position opposite the CPD. These observations suggest that UV mutagenesis may occur in two distinct modes: mutagenesis that occurs directly at the replication fork is the product of base substitutions generated by the action of Pol III HE TLS, whereas mutagenesis resulting from the action of Pol V occurs in the gaps left behind when the replisome performs lesion skipping.

Experimental Procedures

DNA Templates and Proteins—DNA templates containing site-specific lesions were synthesized using M13-JY13 ssDNA and lesion-containing primers [supplemental Table S1] as described previously (22). Replication proteins were purified as described previously (22). Pol III* was isolated from RW644 [BL21(λ DE3) *ApolB::spec* *AdinB::zeo*, *AumuDC::erm*] (a gift of Roger Woodgate, NICHD) harboring the plasmid pHOC 2.6.1 (a gift from C. McHenry, University of Colorado) as described (38). Oligonucleotides were from Integrated DNA Technologies and GeneLink. DNA substrates for primer extension assays were made by annealing oligonucleotides (Table S1) at 90 °C for 2 min followed by slow cooling to room temperature. Primers for primer extension assays were 5'-end-labeled with [γ -³²P]ATP and T4 polynucleotide kinase (New England Biolabs) and purified using G-50 spin columns (GE Healthcare).

Primer Extension—Primer extension reaction mixtures (5 μ l) containing 50 mM HEPES-KOH (pH 8.0), 75 mM K-Glutamate, 10 mM Mg(OAc)₂, 0.1 mg/ml BSA (New England Biolabs), 10 mM DTT, 1 mM ATP, 750 μ M of each dNTP, 10 nM oligonucleotide primer-template, 20 nM Pol III*, 100 nM β ₂, and the 200 nM DnaB and 200 nM DnaC when indicated were

incubated for 5 min at 37 °C. Reactions were quenched by the addition of an equal volume of 20 mM EDTA, 95 % formamide, and 10 mM NaOH. Products were analyzed by electrophoresis at 37 W for 1 h through 20% (19:1 acrylamide:bisacrylamide) polyacrylamide gels using 100 mM Tris-borate (pH 8.3), 2 mM EDTA, and 7 M urea as the gel and running buffer. Products were visualized by phosphorimaging and autoradiography.

Replication

Reactions—Standard replication reaction mixtures (10 μ l) containing 50 mM HEPES-KOH (pH 8.0), 75 mM K-Glutamate, 10 mM Mg(OAc)₂, 0.1 mg/ml BSA (New England Biolabs), 10 mM DTT, 1 mM ATP, 200 μ M each of CTP, GTP, and UTP, 40 μ M of each dNTP, 1 μ M SSB, 8 nM Tus, 20 nM DNA gyrase, 140 nM DnaA, 200 nM DnaB, 200 nM DnaC, 320 nM DnaG, 25 nM HU, 30 nM β ₂, 20 nM Pol III*, and 2 nM DNA template were incubated for 8 min at 37 °C. [α -³²P]dGTP radiolabel was included at 4000 cpm/pmol. To test the effect of elevated nucleotides, an additional 750 μ M of the indicated dNTPs were added. Reactions containing limiting primase were performed in the presence of 10 nM DnaG. Reactions were quenched by addition of an equal volume of stop buffer containing 50 mM HEPES-KOH (pH 8.0), 75 mM K-Glutamate, 10 mM Mg(OAc)₂, 0.1 mg/ml BSA (New England Biolabs), 10 mM DTT, 10 mM AMP-PNP, and 1 mM 2',3'-dideoxyribonucleoside 5'-triphosphates, followed by digestion by EcoRI (4 units) and PvuI (4 units) (New England Biolabs) for 15 min at 37 °C. Products were analyzed by gel electrophoresis as described (22) using native 0.8% agarose gels or 0.6% alkaline agarose gels followed by autoradiography or phosphorimaging.

Pulse-chase replication reactions (20 μ l) were performed as standard replication reactions but initiated in the absence of DNA gyrase and radiolabel. After 1 min of incubation at 37 °C, EcoRI (12 units) was added together with [α -³²P]dGTP and replication was allowed to proceed. After 1 min, a 100-fold excess of cold dGTP was added with or without additional dNTPs and the reactions were quenched after various periods of time as described above and digested by PvuI. Products were analyzed by both native and denaturing gel electrophoresis as described above.

Deep Sequencing—Replication reactions (30 μ l) were carried out as described above in the presence of an additional 750 μ M dATP and dCTP and products were digested with EcoRI, PvuI, and DpnI (40 units) (New England Biolabs). Products were separated using native 0.8% agarose gel electrophoresis and full-length, leading-strand products (22) were isolated using a gel extraction kit (Qiagen). The nascent full-length leading strand was amplified by PCR in two steps using Phusion polymerase (New England Biolabs). PCR products were prepared for deep sequencing and DNA sequences were determined using an Ion PGM by the Sloan Kettering Integrated Genomics Core Facility. The data were analyzed using SAMtools (47) and Integrated Genomics Viewer (48). The error frequency for a specific position was calculated as the fraction of correct reads subtracted from 1. Base substitution frequencies

were calculated as the number of incorrect base reads divided by the number of total base reads at each position.

Conflict of Interest—The authors declare that they have no conflict of interest with the contents of this article.

Author Contributions—PN and KM designed the study, GC made the original observations, PN did all the experiments, PN and KM wrote the manuscript.

²The abbreviations used are: TLS, trans-lesion synthesis; DNA Pol, DNA polymerase; Pol III HE, the DNA Polymerase III holoenzyme; CPD, cyclopymidine dimer; THF, tetrahydrofuran; p/t, primer-template.

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Figure Legends

FIGURE 1. The Pol III HE is capable of TLS. *A*, Map of the plasmid template used in the replication reactions and the position of lesions in each template. Standard replication reactions are incubated in the presence of DNA gyrase for 8 min. Additional replication is inhibited by the addition of AMP-PNP and ddNTPs. The products are digested with EcoRI and PvuI. Products formed with an undamaged template are equal length leading- and lagging-strand sister duplexes. With a damage template several types of products can form: a stalled fork; if lesion skipping occurs the leading-strand sister duplex will contain a

gap between the stall in the nascent leading strand and the point of restart, whereas the lagging-strand sister duplex will be complete; if uncoupled unwinding occurs concomitant with lagging-strand synthesis but in the absence of continued leading-strand synthesis, the leading-strand sister duplex will be incomplete whereas the lagging-strand sister duplex will be complete; if TLS occurs, both the leading- and lagging-strand sister duplexes will be complete with the former carrying a full-length nascent leading strand. Also shown are the sequences at the damage site of the templates used. *B*, TLS by the replisome. Standard replication reactions using the indicated template DNAs and either in the presence (750 μ M) or absence of additional dNTPs were analyzed by electrophoresis through denaturing alkaline agarose gels as described under Experimental Procedures. The extent of replication as a fraction of template utilization (completely replicated templates divided by total template in the reaction), calculated from the extent of incorporation of radioactive precursor into acid-insoluble product, is shown below each lane. *C*, Leading- and lagging-strand synthesis remain equivalent during TLS replication. Standard replication reactions using the indicated templates and either in the presence or absence of elevated concentrations of dNTPs were analyzed by native agarose gel electrophoresis as described under Experimental Procedures. *D*, Extent of TLS on the damage templates. Shown are the mean and standard deviations from five experiments. UN, undamaged template; CPD, CPD-containing template; THF1, THF2, and THF3, THF-containing templates; FL, full length.

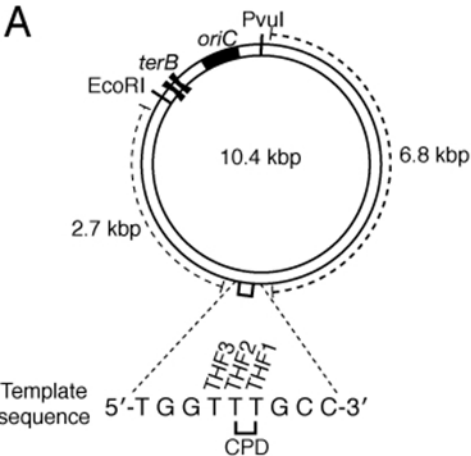
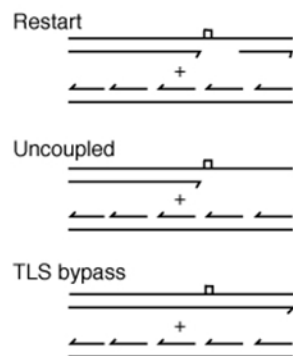
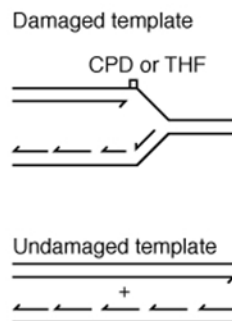
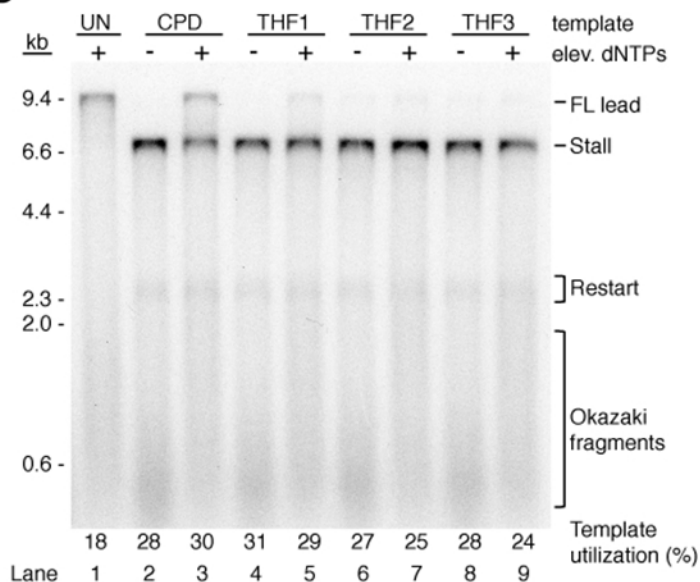
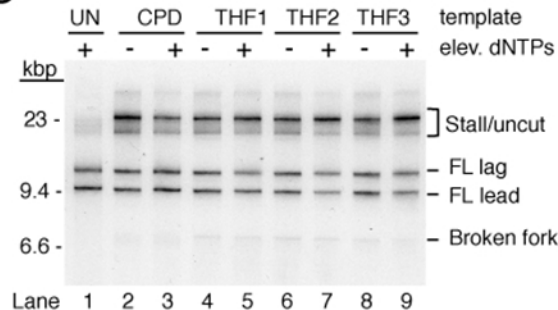
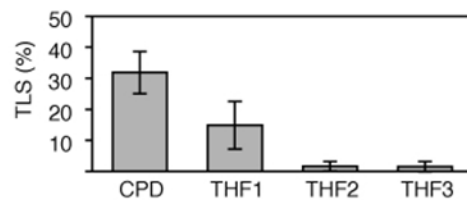
FIGURE 2. Nucleotide requirements for lesion bypass. *A*, Standard replication reactions where the concentration of the indicated nucleotides were increased to 790 μ M with either the CPD or THF1 template were analyzed by denaturing alkaline agarose gel electrophoresis. The extent of TLS is shown below each lane. *B*, Standard replication reactions where the concentration of the indicated combinations of nucleotides were increased to 790 μ M with either the CPD or THF1 template were analyzed by denaturing alkaline agarose gel electrophoresis. The extent of TLS is shown below each lane. AA denotes a doubling of the concentration of dATP.

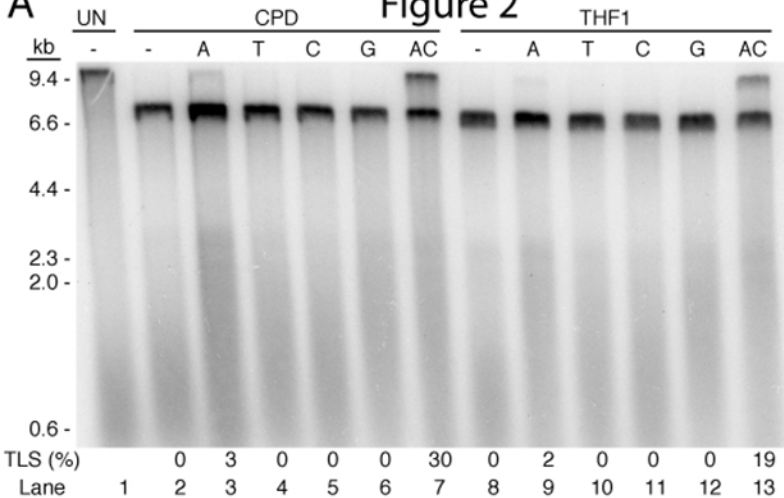
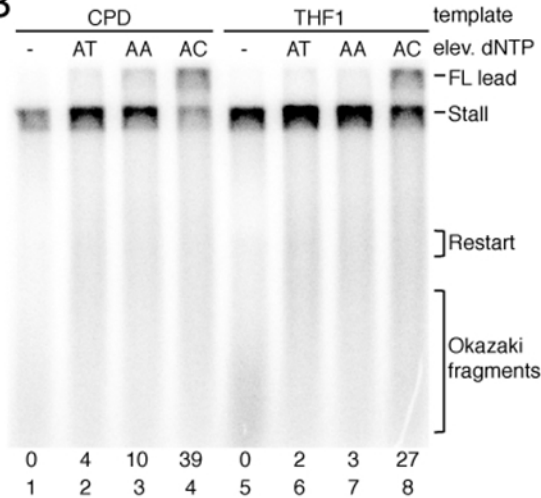
FIGURE 3. Lesion bypass is directly proportional to nucleotide concentration. *A*, Standard replication reactions with the indicated total concentrations of dATP and dCTP were analyzed by denaturing agarose gel electrophoresis. *B*, Quantification of the results shown in *panel A*. Shown is the mean from two experiments.

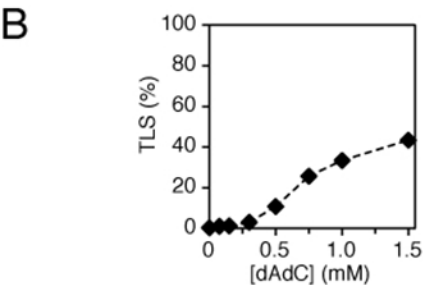
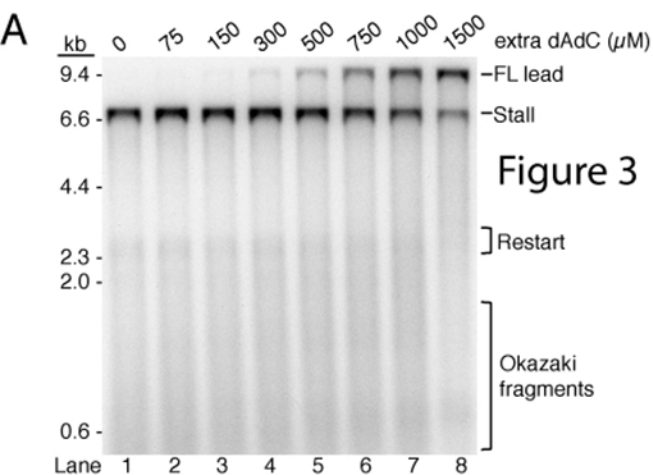
FIGURE 4. The stalled leading strand is extended directly by TLS. *A*, Pulse-chase, EcoRI run-off replication reactions were performed either in the absence or presence of elevated concentrations of dATP and dCTP using the CPD template as described under Experimental Procedures. In these reactions, replication is initiated in the absence of DNA gyrase and allowed to proceed for 1 min when the replication forks stall because of the accumulation of positive supercoils. The template is then digested with EcoRI in less than 10 s at the same time as [α - 32 P]dGTP is added. After 1 min, the radiolabel is chased with an 100-fold excess of cold nucleotide. Time points are as indicated post-chase. The products were analyzed by electrophoresis through either denaturing alkaline agarose gels (top) or native agarose gels (bottom). *B*, Lane traces of the 1, 3, and 6 min lanes in the presence of elevated dATP and dCTP in *panel A*, denaturing.

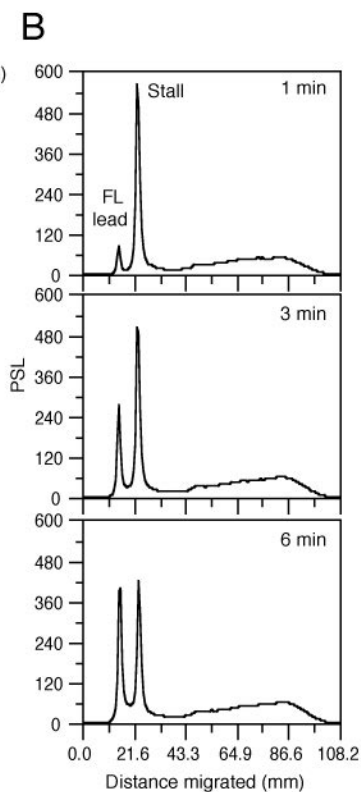
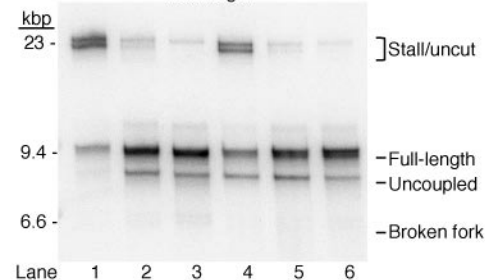
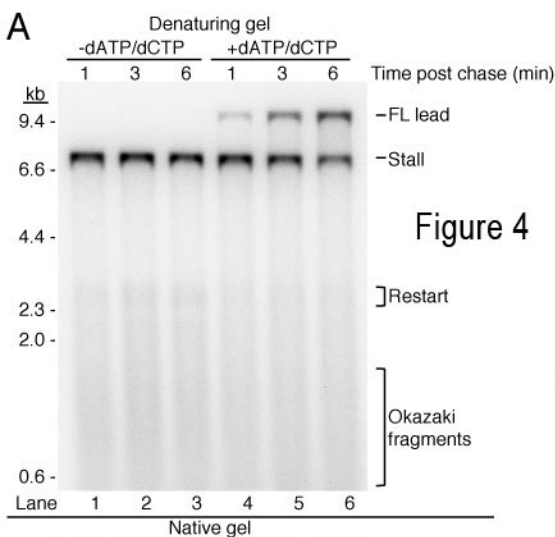
FIGURE 5. The extent of TLS is inversely related to the priming frequency on the leading-strand template downstream of the lesion. *A*, Standard replication reactions (no elevated nucleotide) were performed in the presence of the indicated concentrations of either wild-type or variant DnaG as indicated. Products were analyzed by electrophoresis through denaturing alkaline agarose gels. *B*, Extent of TLS on damaged templates in the presence of 10 nM DnaG and either standard dNTP concentrations or elevated dATP and dCTP. Shown are the mean and standard deviation from three experiments.

FIGURE 6. The Pol III HE alone will not bypass template damage. Primer extension reactions either in the absence or presence of the DnaB helicase using the indicated templates were performed as described under Experimental Procedures. Products were analyzed by electrophoreses through a denaturing 20% polyacrylamide gel. The ** denotes the position of the CPD at the +4 and +5 positions. The THF is at the +4 position.

A**Figure 1****B****C****D**

A**Figure 2****B**





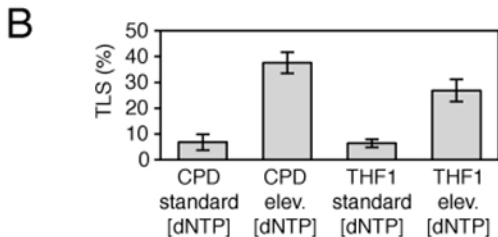
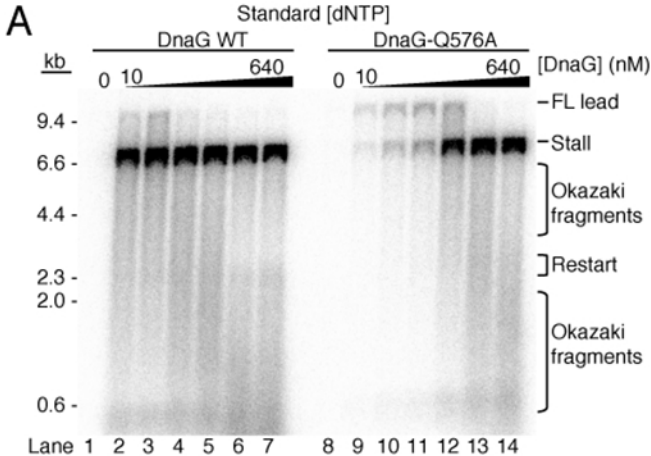
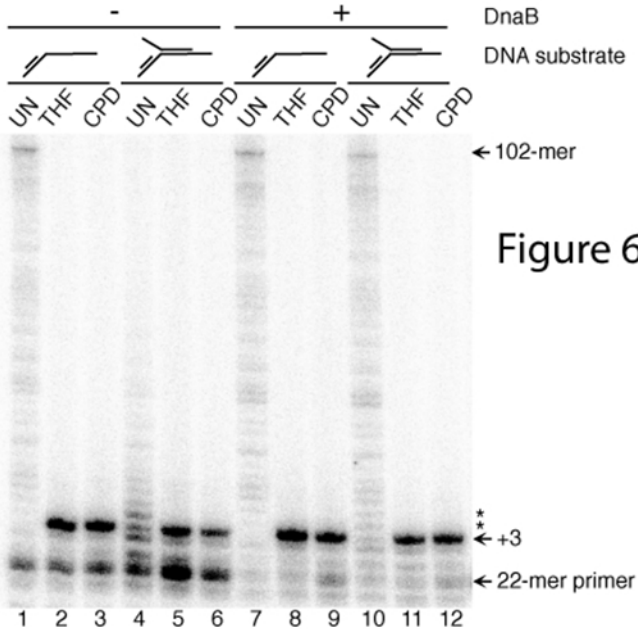


Figure 5



Replisome-mediated Translesion Synthesis by a Cellular Replicase

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J. Biol. Chem. published online June 22, 2017

Access the most updated version of this article at doi: [10.1074/jbc.M117.800441](https://doi.org/10.1074/jbc.M117.800441)

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