Two components of DNA replication-dependent LexA cleavage

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Induction of the SOS response, a cellular system triggered by DNA damage in bacteria, depends on DNA replication for the generation of the SOS signal, ssDNA. RecA binds to ssDNA, forming filaments that stimulate proteolytic cleavage of the LexA transcriptional repressor, allowing expression of > 40 gene products involved in DNA repair and cell cycle regulation. Here, using a DNA replication system reconstituted in vitro in tandem with a LexA cleavage assay, we studied LexA cleavage during DNA replication of both undamaged and base-damaged templates. Only a ssDNA–RecA filament supported LexA cleavage. Surprisingly, replication of an undamaged template supported levels of LexA cleavage like that induced by a template carrying two site-specific cyclobutane pyrimidine dimers. We found that two processes generate ssDNA that could support LexA cleavage. 1) During unperturbed replication, single-stranded regions formed because of stochastic uncoupling of the leading-strand DNA polymerase from the replication fork DNA helicase, and 2) on the damaged template, nascent leading-strand gaps were generated by replisome lesion skipping. The two pathways differed in that RecF stimulated LexA cleavage during replication of the damaged template, but not normal replication. RecF appears to facilitate RecA filament formation on the leading-strand ssDNA gaps generated by replisome lesion skipping.

The integrity of genomic DNA of all organisms is threatened both by environmental and endogenous damaging agents. Bacteria, including Escherichia coli, evolved an inducible system capable of dealing with DNA damage in a coordinated manner known as the SOS response (most recently reviewed in (1)). Activation of the SOS response, which requires DNA replication (2), leads to expression of over forty genes (1, 3–5) that are induced sequentially, transitioning from an error-free to a mutagenic, error-prone DNA repair response (5, 6). E. coli replication initiates at a single origin and proceeds bidirectionally (see review by McHenry (7)). The DnaB helicase unwinds the parental duplex in the 5′ → 3′ direction, traveling on the lagging-strand template. The single-stranded (ss) DNA is immediately sequestered by SSB (ssDNA-binding protein). The primase, DnaG, synthesizes short RNA primers for the initiation of Okazaki fragments on the nascent lagging strand. The DnaX complex, a component of the DNA Polymerase III Holoenzyme (Pol III HE) loads the processivity clamp, β, and the polymerase III core onto the primers. Multiple contacts between replisome components assure optimal DNA synthesis rate and processivity.

Recent studies show that the replication process is dynamic. The leading- and lagging-strand polymerases do not act in a coordinated fashion, but instead pause stochastically and display a wide distribution of polymerization rates (8). During polymerase pauses, the helicase continues to unwind DNA at a substantially reduced rate (8, 9). The decrease in unwinding speed ensures that the transient gaps forming on the DNA template are promptly filled in by the Pol III HE. However, in vivo, replication forks often stall because of encounters with template damage, transcribing RNA polymerases, proteins covalently bound to DNA, or increased template supercoiling. Multiple mechanisms ensure lesion bypass and reactivation of stalled forks (10). Replication of DNA containing lesions was shown to lead to an accumulation of ssDNA, a signal for initiation of the SOS response (2). Two mechanisms are likely to account for generation of the ssDNA signal: 1) Continued unwinding of the parental duplex after the polymerase stalls (11–13), and 2) formation of a ssDNA gap as a result of re-priming downstream of the lesion followed by replisome skipping (14–17).

The recombinase RecA together with ATP binds ssDNA, forming a ternary complex called activated RecA (RecA*) (18). RecA* serves as a co-protease, facilitating self-cleavage of the LexA repressor, thereby inducing the SOS response (19, 20). Late during the SOS response, RecA* is also required for formation and activation of the DNA polymerase V-mutase complex, an error-prone polymerase capable of translesion synthesis (21, 22). The third main role of RecA* is its involvement in error-free DNA repair at ss gaps, double-strand (ds) breaks, and collapsed replication forks through homologous recombination (23, 24). RecA filament assembly on ssDNA is rapid and bidirectional, although it proceeds preferentially in the 5′ → 3′ direction (25, 26). RecA filament assembly is also possible on dsDNA, but occurs more slowly than on ssDNA (27, 28). SSB inhibits RecA filament nucleation on ssDNA (29) but improves extension of the filament by denaturing secondary structure (30). Both RecA nucleation and filament growth are repressed at physiological conditions (25) and require assistance from the RecA-loading proteins, RecF, RecO, and RecR (31–33). A recent study also showed direct displacement of SSB by RecA, but this process is slow and the interaction between SSB and RecA unstable (34).

The RecF, RecO, and RecR (RecFOR) proteins belong to the same genetic pathway, but there is no evidence for RecFOR
complex formation in vivo (25, 35). RecO binds to RecR and the C-terminus of SSB, altering the ssDNA-SSB complex, and, without displacing SSB, facilitates nucleation and growth of the RecA filament (25, 31, 36). RecR itself does not bind to ssDNA, dsDNA, RecA, or SSB, but by interacting with RecO enhances its affinity for SSB-ssDNA complexes (31, 37). RecF preferentially binds gapped DNA (38) and the RecFOR proteins recognize the 5′-end of a dsDNA-ssDNA/RNA junction within the gap (32, 33). Additionally, the RecFR complex binds the 3′-end of a dsDNA-ssDNA junction, halting the growth of the RecA filament beyond it (39). RecF increases RecOR-dependent RecA filament nucleation (25) and stimulates extension past DNA damage by the DNA Polymerase V mutasome (40). However, other studies reported either neutral or inhibitory effects of RecF on RecOR complex function (37, 41–43). It is likely that RecOR and RecFOR constitute two separate RecA loading pathways in E. coli (42), with RecF and RecO competing for binding to RecR (44).

A monomer of the SOS repressor LexA is composed of two domains connected by a flexible linker (45). The N-terminal domain is responsible for binding to DNA, while the C-terminal domain is required for dimerization and self-cleavage (45). Functional LexA is a homodimer that binds to imperfect palindromic operator sequences of SOS genes (46, 47). Approximately 20% of LexA present in cells is thought to be free in the cytoplasm (2) and is the initial target of RecA*, which induces a conformational change in LexA that promotes self-cleavage (19). Current models predict that only one of the subunits in a LexA dimer binds in the deep helical groove of the RecA filament, contacting seven consecutive RecA molecules (48, 49). DNA-bound LexA is unable to interact with RecA* and must dissociate before cleavage (49–51). Differences in the time of expression of SOS genes depend on the LexA binding equilibria to the operators and appear only at high levels of DNA damage, becoming more exacerbated with increasing doses of clastogens (6). Following DNA damage repair and disappearance of the SOS-inducing signal, intact LexA accumulates and the system resets.

We used a reconstituted DNA replication system in tandem with a LexA cleavage assay to show that the SOS-inducing signal, ssDNA, is generated by two related processes: 1) The slow unwinding of the template without concurrent DNA synthesis, resulting from the stochastic pausing of the leading-strand polymerase even in the absence of template DNA damage, and 2) the generation of ss gaps on templates carrying base damage that occurs because of replisome skipping over the DNA lesion. The former process is not stimulated by RecF, whereas the latter process is.

Results
DNA effector requirements for LexA cleavage
ssDNA is the signal for the initiation of SOS response (2), but a RecA-dsDNA filament was also found to mediate LexA cleavage (52). Because we wanted to assess LexA cleavage during DNA replication when both ds and ss DNA would be present in the reaction, we sought to clarify the DNA effector requirements for LexA cleavage. We therefore compared the activity of ssDNA- and dsDNA-RecA filaments in supporting LexA cleavage using a 122 nt-long oligonucleotide (oligo), CG306, in both ss and ds form. In case any unannealed ssDNA remained after annealing the oligos, the dsDNA was treated with E. coli exonuclease VII (Exo VII) (53). To measure LexA cleavage, we used a modified LexA protein, ΔN-His-PKA-LexA, where the first 64 amino acids have been substituted with a 6xHis tag followed by a protein kinase A (PKA) tag (19). The N-terminal deletion prevents LexA binding to DNA whereas the PKA tag allows for radio-labelling of the protein (19). Cleavage of [32P]ΔN-His-PKA-LexA occurs between Ala84 and Gly85, as it does for full-length LexA (19, 54), and is analyzed by SDS-PAGE displaying either a 16.9 kDa full-length protein or a 3.7 kDa N-terminal cleavage product. Hereinafter we will refer to ΔN-His-PKA-LexA as LexA.

We monitored LexA cleavage over 4 h in the presence of RecA and either ss or ds oligo. LexA cleavage supported by the ss oligo increased rapidly, plateauing at 90%, whereas cleavage supported by the ds oligo was much slower: In the first 8 min of the reaction, roughly 80-fold more LexA cleavage was supported by the ss oligo compared with the ds oligo (Figs. 1A and B). In addition, the rate of LexA cleavage in the presence of the ds oligo was almost identical to the rate of autodigestion in the absence of any DNA (Figs. 1A and B). LexA autodigestion is stimulated by increased pH (55). At pH 8.0 (our reaction conditions), the t1/2 of LexA is 4 h (55). We conclude that a RecA-dsDNA filament does not stimulate LexA cleavage.

We planned to study the generation of SOS-inducing ssDNA in a reconstituted DNA replication system with M13 phage-derived ds supercoiled DNA templates. Because M13 RF DNA is purified from infected cells that are also producing ss phage DNA, and the synthesis of ds templates in vitro starts with ss M13 phage DNA, we were concerned that our template preparations might contain extraneous ssDNA that would artifactually stimulate LexA cleavage. We therefore tested the extent to which minor contamination of ds DNA preparations with ssDNA could stimulate LexA cleavage. To do so we annealed the CG306 oligos in the presence of a 5 and 10% excess of one oligo and treated a portion of each reaction with Exo VII. We then measured LexA cleavage supported by these ds oligo preparations. DNA from the 5 and 10% oligo excess annealing reactions that were not subjected to Exo VII digestion induced on average 5- and 16-fold greater LexA cleavage than DNA from the respective annealing reactions treated with Exo VII (Figs. 1C, D and E). Moreover, Exo VII digestion reduced LexA cleavage to levels observed when annealing was performed at a 1:1 ratio (Figs. 1C, lanes 4, 6, 8, and D). These data suggest that even a minor contamination of dsDNA with ssDNA can lead to false positive LexA cleavage results.

We tested four different M13-JY13-derived RF DNA preparations for LexA cleavage in the absence of DNA replication (Fig. 2A). The RF preparations used in the assay had been purified by ethidium bromide CsCl density gradient centrifugation followed by velocity sedimentation in 1 M NaCl. Surprisingly, all of the DNA samples induced 24–59% LexA cleavage, which was abolished when Exo VII was included in the reaction mixture (Fig. 2A). Because M13 RF DNA extracted directly from cells supported high LexA cleavage, we also tested our M13
**JY13 DNA templates synthesized in vitro.** Synthesis is performed by annealing a primer to circular ss phage DNA followed by DNA polymerase-catalyzed extension, ligation, supercoiling, and purification by ethidium bromide CsCl density gradient centrifugation, as described previously (17). However, even the synthesized RF DNA supported at least 25% LexA cleavage in the absence of replication (Fig. 2B). We therefore introduced an additional, final step to the dsDNA synthesis protocol, treating the DNA with Exo VII, followed by gel filtration and ethanol precipitation. After this sanitization, synthesized DNA preparations induced only 2–3% LexA cleavage in a 20 min reaction in the absence of DNA replication (Fig. 2B).

We suggest that previous reports of LexA cleavage being supported by dsDNA are likely a result of contamination with ssDNA.

**SSB is a potent inhibitor of RecA-induced LexA cleavage**

ssDNA produced during replication is immediately sequestered by SSB (56). In *E. coli*, accessory RecA-loading factors RecF, RecO, and RecR help form RecA filaments on SSB-coated ssDNA to initiate the SOS response (42). Our standard replication assays include 250 nM SSB tetramer, which is within the range of SSB concentration measured in exponential phase cells (57–59). To test if SSB can prevent LexA cleavage under our experimental conditions, we titrated RecA in the presence of the ss oligo CG306 and SSB. LexA cleavage was reduced to background levels, even when RecA was in nearly 40-fold excess over the SSB tetramer (Fig. 3A). This inhibition could be reversed by the RecA-loading factors RecOR. LexA cleavage was restored to 40–44% of LexA cleavage levels observed in reactions lacking SSB at all RecA concentrations (Fig. 3B). The addition of RecF to RecOR had little effect, generating 33–37% restoration of LexA cleavage (Figs. 3A and B). This was not surprising because RecF helps to load RecA at dsDNA-ssDNA/RNA junctions in gapped DNA (32, 33) and we used a ss oligo. The slight inhibition observed might be a result of RecF sequestering RecR, preventing it from interacting with RecO, thereby reducing the overall loading of RecA onto ssDNA. We conclude that SSB is a potent inhibitor of RecA-mediated LexA cleavage, indicating that any DNA replication-induced cleavage of LexA is likely to require RecFOR.

**Two components of DNA replication-dependent LexA cleavage**

Our templates for DNA replication are based on M13-JY13 (17) and are 10.4 kbp long (Fig. 4A). Replication is initiated at oriC. The counterclockwise-moving fork is blocked after 0.6 kbp by Tus bound to a Ter site, whereas the clockwise-moving fork can proceed unimpeded on the undamaged (UND) template for 9.6 kbp. The 2xCPD template contains two cyclobutane pyrimidine dimers in the leading-strand template 3.5 kbp and 6.8 kbp downstream from oriC. Our previous studies
demonstrated that the replisome could skip over these sites of DNA template damage, restarting leading-strand synthesis downstream and leaving nascent leading-strand gaps behind (14, 17). Digestion of replication products by EcoRI and PvuI allows us to follow the fate of only the clockwise-moving fork by agarose gel electrophoresis.

We compared RecA- and RecFOR-stimulated LexA cleavage in the presence of DNA replication supported by either the UND or 2xCPD templates (Fig. 4). Neither template supported significant DNA replication nor LexA cleavage in the absence of the DNA replication initiator protein DnaA (Figs. 4B, bottom and top panels, lanes 3 and 5; and C). When replication was ongoing, LexA cleavage was observed for both templates, with a surprisingly significant level occurring with the UND template (Figs. 4B, bottom and top panels, lanes 4 and 6; and C). The extent of LexA cleavage in the reaction mixtures was on average about 50% greater for the 2xCPD template visualized on a denaturing agarose gel lacked the leading-strand replication restart species (Fig. 4B, bottom panel, lane 6), indicating that replication did not proceed past the first CPD lesion, unlike what was observed previously (14). We presume that the lack of restart is a result of the presence of the recombination proteins in the reaction. Given this observation, because replication of the UND template was complete, it was clear that we could not compare LexA cleavage levels supported by the two templates directly. We therefore normalized the data to the amount of template replicated, calculated from the incorporation of [32P]dAMP, by assuming complete replication of all 10.4 kbp with the UND template and only 4.1 kbp of replication (the sum of replication fork progression of the counterclockwise- and clockwise-moving forks) with the 2xCPD template. When expressed in this fashion, it was clear there was no significant difference in LexA cleavage supported by the two templates (Fig. 4C).

How to account for the replication-dependent LexA cleavage with the UND template? Because we do not typically include in the reactions the proteins required to join Okazaki fragments, we considered that one source of ssDNA during replication

Figure 2. Exo VII treatment is required to remove ssDNA contamination from dsDNA preparations. A, M13 RF DNA extracted from cells and, B, synthesized from circular ssDNA must be sanitized by Exo VII treatment to remove contaminating ssDNA. A, LexA cleavage reactions (20 min) containing different RF DNA preparations or ss oligo DNA were performed and analyzed as described under Experimental Procedures. All reactions contained 1 μM RecA and, where indicated, 2 U of Exo VII. B, LexA cleavage reactions (20 min) containing synthesized UND and 2xCPD template DNA were performed and analyzed as described under Experimental Procedures. DNA either was or was not treated with Exo VII, gel filtered, and ethanol precipitated prior to use, as indicated. A representative gel is shown. The table in panel B shows the mean ± S.D. (n = 2).

Figure 3. SSB is a potent inhibitor of RecA-induced LexA cleavage. LexA cleavage reactions (20 min) with the ss oligo containing SSB (250 nM tetramer), the indicated concentrations of RecA, and either 200 nM RecO and 500 nM RecR or 25 nM RecF, 200 nM RecO, and 500 nM RecR, as indicated, were performed and analyzed as described under Experimental Procedures. A, a representative gel. B, plot of the data (mean ± S.D., n = 4).

LexA cleavage (%) DNA effector | - Exo VII | + Exo VII
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UND | 29.4 (± 1.6) | 1.9 (± 0.1)
2xCPD | 24.7 (± 2.1) | 3.0 (± 0.2)
could be gaps between Okazaki fragments. However, including RNase HI, DNA polymerase I, and *E. coli* DNA ligase to seal these gaps did not reduce LexA cleavage supported by the UND template (Figs. 5A, top panel, lanes 2, 6; and B). Omission of any one of the sealing components also did not have a significant effect on LexA cleavage (Figs. 5A, top panel, lanes 3–5; and B). Surprisingly, RNase HI was not required for gap sealing. The replication product profile was identical with or without RNase HI when DNA polymerase I and DNA ligase were present (Fig. 5A, bottom panel, lanes 2, 3), suggesting that the 5′ → 3′ exonuclease of DNA polymerase I digested into the nascent DNA past the RNA primer. We conclude that gaps between Okazaki fragments are not the cause of replication-dependent LexA cleavage.

Gaps may also be created during replication because of stochastic changes in polymerization rates and pausing duration of the leading- and lagging-strand polymerases (8). DnaB helicase still unwinds DNA during polymerase pauses, although more slowly (8, 14). This form of uncoupling is therefore a common event during DNA synthesis. The β sliding clamp enhances DNA polymerase III processivity and increases product length (60, 61). In single molecule experiments, median burst synthesis length decreased and median pause length increased when the concentration of β was reduced, suggesting that under these conditions gap formation increased. To understand if ssDNA produced during uncoupling is the source of replication-dependent LexA cleavage, we lowered the concentration of β in the replication reaction to one-fifteenth the standard level. Under these conditions LexA cleavage increased, whereas total replication decreased (Fig. 6A). Only a small fraction of templates replicated fully at the low concentration of β. The wide

Figure 4. DNA replication-dependent LexA cleavage. A, cartoon of the DNA replication template. DNA replication-LexA cleavage reactions (20 min) containing the indicated template DNA (2 nM), 2 μM RecA, 3 μM [32P]DN-His-PKA-LexA, and 25 nM RecF, 200 nM RecO, and 500 nM RecR, as indicated, were performed and analyzed as described under Experimental Procedures. B, top panel, representative LexA cleavage gel, bottom panel, representative denaturing alkaline agarose gel of the DNA replication products. OF, Okazaki fragments. C, tabulation of LexA cleavage (%) versus template replicated (fmol) for three experiments, N. S., not significant.
Enzymatic reactions performed and analyzed as described in the legend to Fig. 4, as well as RNase H, DNA Polymerase I, and Ligase, as indicated, were performed and analyzed as described under Experimental Procedures. A, top panel, representative LexA cleavage gel, bottom panel, representative denaturing alkaline agarose gel of the DNA replication products. B, dot plot of the data (mean ± S.D., n = 3). Distribution of relative LexA cleavage (LexA cleavage/template molecules replicated) suggests that uncoupling is a stochastic process and a 10.4 kbp template might be too short to observe the maximum effect of reduced concentrations of β on LexA cleavage (Fig. 6B). Relative LexA cleavage was inversely correlated with the concentration of β in the replication reaction, increasing exponentially as the β concentration decreased (Fig. 6C). These data suggest that ss gaps are sufficient to support LexA cleavage can form during DNA replication in the absence of any template damage because of the stochastic uncoupling of the DNA polymerases from the DNA helicase.

How then to explain the equivalent levels of LexA cleavage supported by both the UND and 2xCPD templates in the presence of saturating concentrations of β? We considered that cleavage supported by the 2xCPD template was the sum of two processes: ss gaps created by replisome skipping at the DNA lesion and ss gaps created by stochastic uncoupling. We speculated that if this were the case, ss gaps generated by stochastic uncoupling might be small and quite transient and thus less sensitive to the RecA gap-loading activity of RecF than longer, more stable, ss gaps created on the template as a result of replisome skipping past the lesion. We therefore assessed the effect of RecF on the ability of each template to support LexA cleavage at both low and saturating concentrations of β (Fig. 7).

Consistent with this idea, LexA cleavage supported by the UND template was stimulated by RecF at low, but not saturating concentrations of β (Figs. 7A and B). That stochastic uncoupling was occurring at the limiting concentration of β was clear: 1) little full-length, nascent leading strand was generated (Fig. 7A bottom panel, lanes 3 and 4), and 2) decreased lagging strand—compare with leading strand—synthesis was apparent by native agarose gel electrophoresis (Fig. 7C). On the other hand, LexA cleavage supported by the 2xCPD template was equally stimulated by RecF at both concentrations of β (Figs. 7A and B), even though leading- and lagging-strand synthesis appeared equivalent at the saturating concentration of β (Fig. 7C). Because the presence of RecF favors loading of RecA into the 5′-edge of a gap (32, 33), these data suggest that during DNA replication on the UND template, few or no such gaps are present, whereas the opposite is the case for replication on the 2xCPD template, where a ss gap generated by replisome skipping at the CPD supports LexA cleavage.

**Discussion**

The generation of ssDNA in cells, a trigger for the SOS response, requires active replication forks (2). Two possible sources of ssDNA have been suggested: 1) polymerase uncoupling, leading to DNA helicase unwinding without concurrent DNA synthesis, and 2) replisome skipping over DNA lesions, leaving behind ssDNA gaps (15). In this study we examined DNA replication concomitant with LexA cleavage to understand the requirements for the onset of the SOS response. Only a RecA-ssDNA filament could support LexA cleavage, with apparent stimulation of cleavage by a RecA-dsDNA filament attributable to either autodigestion or trace contamination of dsDNA preparations with ssDNA (Figs. 1 and 2). All dsDNA tested had to be sanitized by treatment with Exo VII to remove these trace contaminations. Furthermore, we found that SSB inhibited RecA filament formation in the absence of Rec(F)OR, even when RecA was in considerable excess and despite reaction conditions previously thought to allow RecA displacement of SSB from ssDNA (62) (Fig. 3).

Active DNA replication was necessary to induce substantial LexA cleavage (Fig. 4). As anticipated, we observed an increase in LexA cleavage during replication of the damaged 2xCPD template where we expected gaps to occur in the nascent leading strand because of replisome skipping over the lesion. Surprisingly, however, we also observed significant DNA replication-dependent LexA cleavage supported by the undamaged template. We initially considered that the ss gaps required on
the UND template might arise between unsealed Okazaki fragments, but inclusion of the proteins necessary to seal the fragments did not suppress LexA cleavage (Fig. 5).

The source of the gaps on the UND template was revealed by the observation that LexA cleavage was inversely proportional to the concentration of \( \beta \) used during replication (Fig. 6). The presence of \( \beta \) stimulates DNA Pol III activity, increasing product length and processivity (60). In agreement with this, we observed a reduction of template molecules with full-length, nascent leading strands and a decrease in total DNA synthesis at lower concentrations of \( \beta \). We have shown that both the leading- and lagging-strand polymerases have a natural tendency for pausing, leading to effective helicase-polymerase uncoupling (8). As DnaB continues unwinding, albeit at a reduced rate, stretches of ssDNA are formed (8, 9). In these single-molecule studies of leading-strand rolling-circle replication, omission of \( \beta \) during replication (after initiation complexes had been formed) led to the emergence of a subpopulation of replisomes with reduced synthesis rates and increased pausing (8), suggesting that some of the polymerase pauses on the leading strand stem from the loss of interaction between polymerase and \( \beta \). Thus, it is likely that stretches of ssDNA arise during replication of the UND template (and therefore during unperturbed DNA replication) because of helicase-polymerase uncoupling.

We noted that LexA cleavage on the UND template, however, was only stimulated by RecF when the concentration of \( \beta \) was limiting (Fig. 7). This may reflect different events during leading- and lagging-strand synthesis. The formation of RecA filaments on the lagging-strand template of UND could be explained by the action of RecF combined with the insufficient availability of \( \beta \). New primers synthesized by DnaG will not be occupied immediately by \( \beta \), enabling RecF to efficiently direct RecA to the 5′-end of gaps formed between the 3′-end of an Okazaki fragment and the 5′-end of the next primer, resulting in formation of RecA filaments. It has been demonstrated that RecF will bind to a ss gap carrying an RNA primer at the 5′-end (33). The RNA primers used to initiate Okazaki fragment synthesis are 10–12 nucleotides long (63), possibly too short to form the stable edge of a gap under these circumstances.
However, we have demonstrated that primer length increases in the absence of interaction between the Pol III HE and DnaG (64), as would occur when β availability is limited, and thus think it likely that the primers will be stably bound to the DNA template. The effect of RecF on the formation of RecA filaments on the leading-strand template is more problematic. The low proportion of full-length, nascent, leading strands during UND replication suggests that in the absence of sufficient levels of β, the synthesis rate of the leading-strand polymerase decreases and the enzyme pauses often, as shown before (8). Uncoupling of the helicase from the leading-strand polymerase will produce ssDNA gaps, but without an obvious 5’-end. Thus, RecF would not be able to improve the formation of RecA filament unless re-priming by DnaG occurs on the leading-strand template. We suspect that such re-priming does not occur frequently enough during stochastic uncoupling to generate the 5’-end of the gap, as opposed to during lesion skipping (discussed below). In our single-molecule experiments (8), pausing and uncoupling occurred at saturating concentrations of DnaG and β, yet the average continuous leading-strand product was 68 knt long. At saturating concentrations of β, LexA cleavage on the UND template was not stimulated by RecF (Fig. 7). Given that sealing of Okazaki fragments did not affect LexA cleavage and the argument above, this observation points to uncoupling of the leading-strand polymerase from DnaB during unperturbed DNA replication as the source of the ssDNA (Figs. 8A and B).

The ssDNA generated by the mechanisms discussed above presumably results in a background level of LexA cleavage insufficient to trigger the SOS response. One might argue, then, either that stochastic uncoupling occurs less frequently in vivo than is observed in vitro or that ss gaps generated by stochastic uncoupling are filled in very rapidly, suppressing potential RecA filament formation. Template DNA damage is required to induce the SOS response (2). LexA cleavage supported by replication of the 2xCPD template was stimulated by RecF at both limiting and saturating concentrations of β (Fig. 7). By the reasoning above, this again points to gap formation in the

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**Figure 7. RecF-stimulation of LexA cleavage differentiates stochastic uncoupling from lesion skipping.** DNA replication-LexA cleavage reactions containing the indicated templates and concentrations of β, and either 200 nM RecO and 500 nM RecR or 25 nM RecF, 200 nM RecO, and 500 nM RecR, as indicated, were performed and analyzed as described under Experimental Procedures. A, top panel, representative LexA cleavage gel, bottom panel, representative denaturing alkaline agarose gel of the DNA replication products. B, dot plot of the ratio of LexA cleavage (%) relative to template replicated (fmol) in reactions containing RecFOR to reactions containing RecOR (mean ± S.D., n = 6; nonparametric two-tailed Mann-Whitney test, **, p < 0.01, ns, not significant). C, native agarose gel visualization of the relative extents of nascent leading- and lagging-strand synthesis for the replication reactions as described. DNA products have been digested with EcoRI and PvuI. Because lagging-strand synthesis does not proceed all the way to the EcoRI site, this separates the leading- and lagging-strand sister molecules (17).
LexA cleavage during DNA replication

A

OF RNA primer
nascent leading strand

helicase

B

undamaged DNA

rare stochastic pause

3’

RecA

RecOR

C

base damaged DNA

stall

3’

RecF

Figure 8. Two components of DNA replication-dependent LexA cleavage. A, a schematic of a replication fork showing the nascent leading- and lagging-strand DNA and the position of the DnaB helicase relative to the fork. B, during a stochastic uncoupling event, the leading-strand polymerase pauses, but the helicase continues to unwind the parental DNA at a reduced rate, generating a transient ss region on the leading-strand sister. RecOR can nucleate RecA filament formation downstream of the paused leading-strand polymerase, but because there is no 5’-ds edge on the ssDNA RecF has little effect. C, when the leading-strand polymerase encounters a DNA lesion such as a cyclobutane pyrimidine dimer, lesion skipping occurs whereby the stalled leading-strand polymerase cycles downstream past the damage to a new primer synthesized by DnaG on the leading-strand template, thereby restarting DNA synthesis. The ss gap that is formed is now a substrate for RecFOR, which will nucleate a RecA filament at the 5’-end of the gap.

Fundamentally, replisome skipping of DNA lesions is a natural consequence of the stochastic uncoupling of the leading-strand DNA polymerase from the DNA helicase (10). Thus, we have outlined two DNA replication-dependent pathways that can generate ssDNA available for RecA filament formation leading to SOS induction.

Experimental Procedures

DNAs

Oligonucleotides—CG306 (5’-GGATCCCCGGGTACCAGAGCTGATCGCCTTTACCCGCTGAGATTTGCTGACAGGTCGCTGCTGATTTGAGCCGCCAGGGCGGAAATCCGTGTTATGTTGCTCGGAAATCGCGG-3’) and its reverse-complement, CG306rc, were annealed at 1 μM each (or 5 and 10% excess of CG306, as indicated) in reaction mixtures containing 100 mM NaCl and 5 mM EDTA by heating to 90°C and slowly cooling to room temperature. Part of the DNA was recovered by ethanol precipitation and resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Where indicated, the other part of the annealing reaction was further processed as follows: Duplex oligo (300 nM) was treated with Exo VII (5 U, NEB) in a reaction mixture (50 μl) containing Exo VII buffer (NEB) for 1 h at 37°C. The reaction mixture was diluted with an equal volume of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, SDS and protease K were added to final concentrations of 1% and 0.2 mg/ml, respectively, and the incubation continued for 30 min at 37°C. DNA was recovered by ethanol precipitation after extraction with phenol-CHCl3 and resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA.

Preparation of bacteriophage M13 RF DNA

ds RF DNAs were prepared from 8 liters of infected XL1-Blue Δ tus cells as described (67) with the following modifications: The cell pellet was washed in 160 ml of 10 mM Tris-Cl pH 7.5, 1 mM EDTA, 10 mM NaCl. RF DNA was isolated using the Alkaline Lysis with SDS: Maxiprep protocol (67). DNA was resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and treated twice with RNase A (20 μg/ml) for 30 min at 37°C and recovered by ethanol precipitation after extraction with phenol-CHCl3. RF DNA was then purified by ethidium bromide CsCl density gradient centrifugation followed by velocity sedimentation through 5–20% sucrose gradients containing 1 M NaCl.

DNA template synthesis

DNA templates were synthesized from M13-JY13 ssDNA as described previously (14, 17). The 2xCPD template corresponds to CPD-A + C in (14) and was synthesized by annealing two oligonucleotides with CPD modifications (TT): WF119 (CPD modification is 3472 bp from the PvuI site), 5’-CATTTAAGGGTAA (TT)CTACCCGTCACGG-3’ and 5903CPD (CPD modification is 6744 bp from the PvuI site), 5’-GCTTACGCTGTTGCTTCCGAAATCGCGG-3’). Undamaged template (UND) was synthesized using an oligo with a sequence identical to 5903CPD, but without any modifications. Following DNA synthesis, template (30 μg) was treated (200 μl reaction mixture) with 40 U of Exo VII as above and then gel filtered through a 10 ml Sepharose
PKA-LexA was purified and radiolabeled with RecF, RecO, and RecR were purified as described (72). Each EcoRI-HF (NEB) and PvuI-HF (NEB). EDTA was then added to a final concentration of 30 mM and the DNA products analyzed by gel electrophoresis as described (17) using 0.6% alkaline agarose and 0.8% native agarose gels followed by autoradiography and phosphorimaging.

**Data availability**

Raw data are held by the authors and is available on request.

**Author contributions**—K. K. M. and K. J. M. conceptualization; K. K. M. and K. J. M. data curation; K. K. M. and K. J. M. formal analysis; K. K. M. investigation; K. K. M. and K. J. M. methodology; K. K. M. writing-original draft; K. K. M. and K. J. M. writing-review and editing; K. J. M. resources; K. J. M. supervision; K. J. M. funding acquisition; K. J. M. project administration.

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**Abbreviations**—The abbreviations used are: ss, single-stranded; SSB, single-stranded DNA-binding protein; Pol III HE, DNA polymerase III holoenzyme; Pol III*, Pol III HE lacking β; ds, double-stranded; oligo, oligonucleotide; CPD, cyclobutane pyrimidine dimer.

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