Genetic and Biochemical Characterization of a Novel umuD Mutation: Insights into a Mechanism for UmuD Self-Cleavage

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Most translesion DNA synthesis (TLS) in Escherichia coli is dependent upon the products of the umuDC genes, which encode a DNA polymerase, DNA polymerase V, with the unique ability to replicate over a variety of DNA lesions, including cyclobutane dimers and abasic sites. The UmuD protein is activated for its role in TLS by a RecA–single-stranded DNA (ssDNA)-facilitated self-cleavage event that serves to remove its amino-terminal 24 residues to yield UmuD9. We have used site-directed mutagenesis to construct derivatives of UmuD and UmuD9 with glycines in place of leucine-101 and arginine-102. These residues are extremely well conserved among the UmuD-like proteins involved in mutagenesis but are poorly conserved among the structurally related LexA-like transcriptional repressor proteins. Based on both the crystal and solution structures of the UmuD9 homodimer, these residues are part of a solvent-exposed loop. Our genetic and biochemical characterizations of these mutant UmuD and UmuD9 proteins indicate that while leucine-101 and arginine-102 are critical for the RecA-ssDNA-facilitated self-cleavage of UmuD, they serve only a minimal role in enabling TLS. These results, and others, suggest that the interaction of RecA-ssDNA with leucine-101 and arginine-102, together with numerous other contacts between UmuD9 and the RecA-ssDNA nucleoprotein filaments, serves to realign lysine-97 relative to serine-60, thereby activating UmuD9 for self-cleavage.

When an organism’s DNA replication machinery encounters a lesion in the DNA that, for a variety of reasons, was not repaired by accurate repair pathways, it stalls, leading to one of two possible outcomes: (i) damage avoidance, a poorly understood set of processes, including daughter strand gap repair, that appear to utilize the information in the newly synthesized daughter strand of the DNA duplex to somehow bypass the lesion (17) or (ii) translesion synthesis (TLS), in which a specialized DNA polymerase is recruited for bypassing the damaged site (17, 21, 67, 69). The latter pathway is potentially mutagenic due to the miscoding or noncoding nature of the DNA lesion (17).

TLS in E. coli is dependent on the umuDC and recA gene products (17, 23, 61). The umuDC operon encodes a DNA polymerase, DNA polymerase V, with the unique ability to replicate over particular types of DNA lesions, including abasic sites and thymine-thymine cyclobutane dimers (55, 64, 65). Recent work indicates that UmuC is the founding member of a diverse and ubiquitous family of DNA polymerases capable of copying imperfect templates (16, 18, 21, 69). In addition to their role in TLS, the umuDC gene products also participate in cell cycle checkpoint control (41, 46). In response to DNA damage, RecA protein nucleates on single-stranded DNA (ssDNA) that is generated by the cell’s failed attempts to bypass lesions in its genome (17, 57). These RecA-ssDNA nucleoprotein filaments act to mediate the cleavage of the LexA repressor (33, 34). Cleavage of LexA inactivates it as a repressor, leading to the expression of the SOS regulon, a collection of ~30 unlinked genes whose expression is coordinately regulated (11, 17, 25). The umuDC operon is among these ~30 LexA-regulated genes (9, 17). Importantly, UmuD also undergoes RecA-ssDNA-mediated cleavage. This cleavage serves to remove its amino-terminal 24 residues to produce UmuD9 (5, 43, 58). It has been proposed that cleavage of UmuD serves to regulate the two physiological roles of the umuDC gene products so that they act in a temporally ordered fashion (41, 46), first by participating in a DNA damage checkpoint control system and second by participating in TLS.

UmuD is related to three distinct classes of proteins: (i) other UmuD-like proteins involved in mutagenesis, which also undergo RecA-ssDNA-facilitated cleavage (17, 29, 30, 52), (ii) transcriptional repressors belonging to the LexA-like family, which undergo RecA-ssDNA-facilitated cleavage (17, 52), and (iii) signal peptidases (49). Interestingly, despite the fact that UmuD9 and E. coli signal peptidase have little sequence identity apart from their lysine-serine dyad, they have a remarkable degree of structural identity. Comparison of their crystal structures revealed that 69 Cα atoms of UmuD9 are superimposable (with a root mean squared of 1.6 Å) on the E. coli signal peptidase crystal structure (49).

The E. coli UmuD and UmuD9 proteins interact with each other to form homo- and heterodimers (1, 5, 22) and also interact with UmuC (4, 22, 70), RecA-ssDNA nucleoprotein filaments (5, 32), and three components of the replicative DNA polymerase (62). Given the rather small sizes of UmuD and UmuD9, an important question is which part(s) of UmuD and UmuD9 is involved in interaction with each of these other proteins? A detailed understanding of these interactions will be required for a complete understanding of the molecular mechanisms underlying the roles of the umuDC gene products in checkpoint control and TLS. Determination of the crystal (10, 50) and solution (A. E. Ferentz, G. C. Walker, and G. Wagner, unpublished data) structures of the UmuD9 homodimer and genetic studies (39, 45) have identified the resi-
dyes involved in its dimerization interface. Furthermore, biochemical characterizations of single-cysteine derivatives of UmuD and UmuD' by analyzing homo- and heterodimer cross-linking efficiency using thiol-specific cross-linking reagents and by studies of spin-labeled derivatives by electron spin resonance have identified important components of the dimerization interfaces of the UmuD-UmuD' heterodimer and the UmuD2 homodimer (unpublished data). Finally, these single-cysteine derivatives of UmuD have also been used, in conjunction with the thiol-specific, photoactivatable, heterobifunctional cross-linking agent p-azidooiidoacetanilide (71), to identify residues of UmuD able to cross-link efficiently to RecA-ssDNA nucleoprotein filaments (32) (Fig. 1). Taken together, these studies have begun to provide a detailed molecular understanding of the roles of the umuD gene products in regulation of the cell cycle and TLS.

As part of our ongoing effort to better understand how UmuD and UmuD' interact with other proteins to enable checkpoint control and TLS, we have embarked on a site-directed mutational analysis of the umuD gene products (1, 45). Comparison of the deduced amino acid sequences of members of the UmuD-like mutagenesis proteins to those of the LexA-like transcriptional repressor family identified a small number of residues that were well conserved exclusively among the UmuD-like mutagenesis proteins (1) (see Fig. 2). This observation suggested that these might be residues that are important for the biological roles of the umuD gene products rather than for the RecA-ssDNA-facilitated cleavage of UmuD. Mutations affecting most of these highly conserved positions have already been characterized (19, 39, 45). Here we describe our construction and genetic and biochemical characterizations of a UmuD derivative in which two such highly conserved residues, leucine-101 (Leu101) and arginine-102 (Arg102), have been replaced with glycines. Our characterizations of this mutant UmuD protein, which we unexpectedly found to be deficient in RecA-ssDNA-facilitated cleavage, suggest a possible mechanism for how the interaction of UmuD2 with the RecA-ssDNA nucleoprotein filament results in the cleavage of one UmuD molecule by its intradimer partner to yield UmuD' (10, 37).

MATERIALS AND METHODS

Bacteriological techniques. E. coli strains and plasmid DNAs used in this study are described in Table 1. Generalized transduction using P1vir was performed as described previously (40). E. coli was grown in either Luria-Bertani medium or M9 minimal medium (40) as indicated in Fig. 3 and 4. When required, the following antibiotics were used at the indicated concentrations: ampicillin, 150 μg/ml; kanamycin (KAN), 40 μg/ml; tetracycline, 20 μg/ml; rifampin, 50 μg/ml. Bacterial transformation was by the calcium chloride technique (56). Plasmid DNAs were purified using the QIA-spin mini prep kit (Qiagen) per the manufacturer’s recommendations. Missense mutations within the umuD and umud2 coding regions were generated using the Quickchange kit (Stratagene) per the manufacturer’s recommendations, and the nucleotide sequences of all constructs were verified by automated DNA sequence analysis.

Proteins and reagents. The UmuD and UmuD1012 proteins were purified as described previously from 1-liter cultures of BL21(DE3) transformants except that Superose 12 chromatography was omitted (10). The chromatographic characteristics of UmuD1012 were essentially identical to those of the wild-type UmuD throughout its purification, suggesting that it had a native conformation and that the Leu101-to-glycine and Arg102-to-glycine substitutions had at most a minimal effect on the overall structure of the protein. RecA protein was purified as described elsewhere (15). M13mp18 ssDNA was from New England Biolabs, glutaraldehyde and adenosine 5’-(3-thiotriphosphate) were from Sigma, and the Western-Light chemiluminescence kit was from Tropix.

SOS mutagenesis assays and Western blot analysis. SOS mutagenesis activity was measured by either the argE3(Oc)→Arg+ reversion assay as described elsewhere (9) or a rifampin resistance assay. For the rifampin resistance assay, 0.5 ml of cultures grown overnight at 42°C was used to inoculate 25 ml of M9 minimal medium supplemented with 0.4% Casamino Acids, 0.4% glucose, 100 μg of adenine per ml, and KAN. Cultures were grown at 42°C with shaking. We used these growth conditions because they have been previously demonstrated to enhance the coprotease activity of RecA protein (6). When cultures reached an optical density at 595 nm of ~0.6, 6-ml aliquots of each were removed and either irradiated with UV (20 J/m2) or mock irradiated in sterile Pyrex dishes (100 mm by 15 mm). Irradiated and mock-irradiated cultures were then returned to 42°C and grown with aeration to allow the expression of SOS-regulated functions. Forty-five minutes after irradiation, 4-ml aliquots were removed from each culture for determination of their optical density at 595 nm and for preparation of whole-cell lysates to monitor the relative abundance of UmuD (or UmuD1012) and UmuD' (or UmuD1012') by Western blotting as described previously (45, 91).
We employed site-directed mutagenesis to construct a UmuD mutant having glycines in place of Leu101 and Arg102. These residues are highly conserved among the UmuD-like mutagenesis proteins but not among members of the structurally related LexA-like transcriptional repressor family (Fig. 2), a fact that initially suggested to us that these residues might be important for the specific biological roles of the umuD gene products rather than for the RecA-ssDNA-mediated self-cleavage of UmuD. We chose to change Leu101 and Arg102 to glycines instead of alamines because, based on the crystal structure of the UmuD population (10, 50) and the solution structure determined by nuclear magnetic resonance (Ferentz et al., unpublished data), they are located in a solvent-exposed loop of UmuD (Fig. 1 and 2). We chose to analyze the double mutant in this study because the close proximity of Leu101 and Arg102 together with their high degree of conservation among the UmuD-like mutagenesis proteins suggests their mutual participation in a single function(s). Thus, we reasoned that the double mutant would exhibit a more pronounced phenotype.

**TABLE 1. Bacterial strains and plasmid DNAs used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics or comments</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAG12077</td>
<td>Used as the donor for P1 vir-mediated transduction of the crcA280::Tn10 locus</td>
<td>E. coli Genetic Stock Center</td>
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<tr>
<td>BL21(DE3)</td>
<td>Used for overexpression of UmuD and UmuD1012</td>
<td>Novagen</td>
</tr>
<tr>
<td>GW8023</td>
<td>recA-4 lexA-4 ΔumuDC595::cat</td>
<td>47</td>
</tr>
<tr>
<td>GW8024</td>
<td>recA-4 lexA-4ΔumuDC595::cat</td>
<td>47</td>
</tr>
<tr>
<td>GW8025</td>
<td>GW8024; recA441</td>
<td>47</td>
</tr>
<tr>
<td>GW8018</td>
<td>GW8025; lexA4(Def)</td>
<td>47</td>
</tr>
<tr>
<td>GW8040</td>
<td>GW8024; recA430 slick::Tn10</td>
<td>41</td>
</tr>
<tr>
<td>NR9350</td>
<td>recA730 slick::Tn10</td>
<td>R. Schaaper via V. Godoy and M. Fox (12)</td>
</tr>
<tr>
<td>GW8110</td>
<td>GW8024; recA730 slick::Tn10</td>
<td>This work</td>
</tr>
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</table>

Plasmids

- pBR322kan: Ap′ Km′ Te′; pBR322 derivative expressing KAN resistance
- pSE17: Ap′ Km′; pBR322 derivative carrying umuD′ C′
- pSE17-1012: Ap′ Km′; pSE17 derivative carrying umuD1012C′
- pGW3751: Ap′ Km′; pGW3751 derivative carrying umuD1012C′
- pAG98: Ap′; UmuD′ overproducer
- pAG98-1012: Ap′; UmuD1012 overproducer

In vitro cleavage of UmuD reconstituted with purified components. In vitro cleavage of UmuD was reconstituted with purified components essentially as described elsewhere (5). Briefly, reaction mixtures (20 μl) containing the indicated amounts (indicated in Fig. 5) of purified UmuD, UmuD1012, and RecA; 1.7 μM ssDNA 20-mer oligonucleotide; 2.3 mM adenosine 5′-O-(3-thiotriphosphate); 50 mM Tris-HCl (pH 7.5); 100 mM NaCl; 20 mM MgCl2; 0.1 mM EDTA; 0.1 mM dithiothreitol, and 10% glycerol were assembled on ice. Reactions were initiated by incubation at 37°C and were quenched at the indicated times (indicated in Fig. 5) by addition of 0.25 volume of 4× sodium dodecyl sulfate (SDS) sample buffer (200 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 8% SDS, 0.8% bromophenol blue, and 40% glycerol) followed by heating to 95°C for 5 min.

Aliquots of each sample were then electrophoresed in SDS-14% polyacrylamide gels followed by staining with Coomassie blue R-250. Cleavage efficiency was determined by densitometric analysis of the stained gels relative to appropriate standard curves (data not shown) using the Molecular Analyst software package (Bio-Rad). For time course experiments, reaction mixtures (140 μl) containing 24.5 μg of RecA (4.6 μM as a monomer) and 14 μg of either UmuD or UmuD1012 (3.3 μM as a dimer) were assembled on ice and then transferred to 37°C. Twenty-microliter aliquots were removed after 15, 30, 60, 90, and 120 min of incubation at 37°C and quenched as described above. For RecA-ssDNA titrations, the ssDNA concentration was maintained at 1.7 μM. Reaction mixtures (20 μl) contained 0, 0.44, 0.9, 1.75, 3.5, or 7 μg of RecA (0.58 to 9.2 μM as a monomer) and 2 μg of UmuD or UmuD1012 (3.3 μM as a dimer). Incubation was at 37°C for 30 min.

**RESULTS**

umuD1012, but not umuD1012, is active in SOS mutagenesis. We employed site-directed mutagenesis to construct a plasmid-carried umuDC operon that expresses a UmuD derivative having glycines in place of Leu101 and Arg102. These residues are highly conserved among the UmuD-like mutagenesis proteins but not among members of the structurally related LexA-like transcriptional repressor family (Fig. 2), a fact that initially suggested to us that these residues might be important for the specific biological roles of the umuD gene products rather than for the RecA-ssDNA-mediated self-cleavage of UmuD. We chose to change Leu101 and Arg102 to glycines instead of alamines because, based on the crystal structure of the UmuD population (10, 50) and the solution structure determined by nuclear magnetic resonance (Ferentz et al., unpublished data), they are located in a solvent-exposed loop of UmuD (Fig. 1 and 2). We chose to analyze the double mutant in this study because the close proximity of Leu101 and Arg102 together with their high degree of conservation among the UmuD-like mutagenesis proteins suggests their mutual participation in a single function(s). Thus, we reasoned that the double mutant would exhibit a more pronounced phenotype.

**FIG. 2.** Partial amino acid alignment of proteins similar to E. coli UmuD. Shown is the region between amino acids 94 and 108 of UmuD. This figure is modified from references 1 and 51. UmuD-like proteins (10) are grouped separately. The active-site lysine (Lys97) in UmuD. This figure is modified from references 1 and 51. UmuD-like proteins (10) are grouped separately. The active-site lysine (Lys97) in UmuD, which are based on the crystal structure (10), are as well as the recently solved solution structure (Ferentz et al., unpublished data) form a solvent-exposed loop, as well as the corresponding residues in the related proteins, are indicated by the shaded box. Leu101 and Arg102 are represented as white letters. Ec, E. coli; St, Salmonella enterica serovar Typhimurium.

![Image](http://jb.asm.org/)
than either of the single mutants. Finally, for the sake of simplicity, we will ascribe all phenotypes unique to UmuD1012 and/or UmuD’1012 to both substitutions throughout this report.

In the context of this report, the resulting umuD allele will be referred to as umuD1012, and its gene product will be referred to as UmuD1012. In addition, a synthetically engineered plasmid-carried operon that directly expresses the UmuD’ protein together with UmuC (pGW3751) (43) was similarly mutagenized. The resulting umuD’ allele will be referred to as umuD’1012, and its gene product will be referred to as UmuD’1012. It is important to note that the expression of all of these operons was under the control of the native, LexA-regulated umuD”C” promoter.

We first investigated whether UmuD1012 was competent in SOS mutagenesis. The ability of a pBR322 derivative lacking a umuDC operon (pBR322kan) or bearing the wild-type umuDC”C” (pSE117) or the mutant umuD1012C” (pSE117–1012) operon to functionally complement a ΔumuDC host for SOS mutagenesis was assessed using an in vivo assay that quantitated the reversion frequency of the argE3(Oc) allele from an Arg” to an Arg” phenotype following a UV dose (9). Compared to the wild-type umuDC operon, pBR322kan, umuD1012C” was essentially inactive in SOS mutagenesis (Fig. 3). The observed lack of SOS mutagenesis was not due to an instability of the UmuD1012 protein, as immunoblot analysis of whole-cell extracts indicated that it was as abundant as wild-type UmuD following UV irradiation (see below).

UmuD must undergo a posttranslational, RecA-ssDNA-facilitated self-cleavage reaction to activate it for its role in SOS mutagenesis (5, 43, 58). Interestingly, this cleavage reaction can occur in an intermolecular fashion in which the catalytic dyad of one UmuD molecule cleaves in between Cys24 and Gly25 of its intradimer partner (10, 37). Since it was unclear whether the UmuD1012 protein was deficient in cleavage or in its ability to participate in SOS mutagenesis, we tested the ability of the umuD1012C” operon to complement the ΔumuDC strain in SOS mutagenesis. Our observation that the strain expressing the precleaved UmuD’1012 protein together with UmuC was ~60% as active in SOS mutagenesis as the control strain expressing wild-type UmuD’ together with UmuC (Fig. 3) suggests that the proximal cause of the nonmutability of the strain expressing umuD1012C” was a deficiency of the UmuD1012 protein in promoting the TLS process that underlies SOS mutagenesis.

**Table 2.** umuD1012C” confers a more severe cold sensitive phenotype than does umuD”C”

<table>
<thead>
<tr>
<th>GW8018 transformant</th>
<th>umuD allele*</th>
<th>CPU (30°C/42°C)$^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322kan</td>
<td>None</td>
<td>0.9</td>
</tr>
<tr>
<td>pSE117</td>
<td>umuD”</td>
<td>4.6 \times 10^{-4}</td>
</tr>
<tr>
<td>pSE117-1012</td>
<td>umuD1012</td>
<td>2.3 \times 10^{-5}</td>
</tr>
</tbody>
</table>

* pSE117 and pSE117-1012 both carry the wild-type umuC allele.

$^\dagger$ Representative transformants obtained at 42°C were picked and resuspended in 0.8% saline. After serial dilution, aliquots were plated in duplicate onto Luria-Bertani agar supplemented with KAN and then incubated at 30 or 42°C overnight prior to colony counting.
participate in umuDC-mediated cold sensitivity must remain largely intact in the UmuD1012 protein.

**UmuD1012 is defective in RecA-ssDNA-facilitated self-cleavage in vivo.** As a direct test of the possibility that the UmuD1012 protein was defective in RecA-ssDNA-facilitated cleavage, we measured its cleavage in vivo to yield UmuD'1012 following a UV dose of 20 J/m^2_. Cleavage was monitored directly by Western blotting of whole-cell extracts using affinity-purified anti-UmuD-UmuD' antibodies as previously described (45, 46). Preliminary experiments indicated that whereas wild-type UmuD was efficiently cleaved to yield UmuD' after UV irradiation of a recA^+ strain, UmuD1012 was not. We therefore investigated whether cleavage of UmuD1012 could be facilitated in vivo by RecA derivatives whose coprotease activity is altered. For these experiments we used culturing conditions previously demonstrated to enhance the coprotease activity of RecA protein (see Materials and Methods). Plasmids carrying umuD^C' or umuD1012C' were introduced into ΔumuDC derivatives of recA^+, recA430, recA441, or recA730 strains. The recA430 allele encodes a mutant RecA protein containing a glycine-204-to-serine (G204S) substitution that renders it deficient in facilitating self-cleavage of UmuD, LexA, and λcI repressor while leaving it proficient in homologous recombination (24). The recA441 and recA730 alleles encode a common Q38K substitution, with the recA441 allele encoding a V298I substitution as well (26, 68). The recA441 mutant is coprotease constitutive at 42 but not 30°C, while the recA730 mutant is coprotease constitutive at both temperatures (68; reviewed in reference 28). Since the RecA441 mutant is coprotease constitutive at 42°C and since we wanted to maximize the coprotease activity of all RecA derivatives, we grew all cultures, regardless of their genotype, at the permissive temperature of 42°C (see Materials and Methods).

Essentially no UV-induced cleavage of UmuD1012 was observed in the recA^+ strain (Fig. 4A, lane 2), and, as expected, neither UmuD nor UmuD1012 was cleaved in the recA430 strain (Fig. 4A, lanes 3 to 6). In contrast, however, the recA441 and recA730 genetic backgrounds were capable of facilitating cleavage of both UmuD and UmuD1012, with or without UV irradiation (Fig. 4A, lanes 7 to 14), with recA730 being slightly better than recA441 at mediating cleavage of UmuD1012. Nevertheless, this cleavage of UmuD1012 was still far less efficient than that observed for wild-type UmuD (Fig. 4A, compare lanes 9 and 10 and lanes 13 and 14 with lane 1). The ability of the recA441 and recA730 gene products to facilitate the cleavage of UmuD1012 in vivo suggests that the RecA441 and the RecA730 proteins interact with UmuD^2 in such a way that, for them, Leu101 and Arg102 are less important for stimulating the cleavage reaction.

**UmuD1012 is active in SOS mutagenesis in vivo in a recA allele-specific fashion.** In a parallel experiment, we compared the abilities of the UmuD and UmuD1012 proteins to participate in SOS mutagenesis as a function of the recA alleles discussed above. This was done by plating aliquots of the same UV-irradiated cultures used for the analysis of the levels of the UmuD (or umuD1012) gene products onto solid medium containing rifampin. Quantitation of the rifampin-resistant CFU per survivor for each strain allowed a direct comparison of the UmuD and UmuD1012 alleles with respect to their activity in SOS mutagenesis. The presence of the wild-type umuDC operon efficiently complemented the ΔumuDC allele of the host strains, as indicated by the increased frequency of rifampin resistance with all recA alleles tested except for...
recA430, which is defective in promoting cleavage of UmuD (Fig. 4B to E). Furthermore, the frequency of umudC-dependent rifampin resistance was itself enhanced relative to the recA+ strain by recA alleles exhibiting enhanced coprotease activity (i.e., recA441 and recA730 [Fig. 4, compare panels B, D, and E]), as expected (reviewed in references 28 and 66).

Consistent with our earlier observation indicating that UmuD1012 was essentially inactive in SOS mutagenesis in a recA+ genetic background using the argE3(Oc)→Arg+ reversion assay (Fig. 3), umuD1012C was similarly unable to enhance the frequency of rifampin resistance beyond that observed for the ΔumuDC strain lacking a plasmid-carried umuDC operon (Fig. 4B). This inactivity of umuD1012C in SOS mutagenesis correlates with the inability of recA+ to facilitate self-cleavage of UmuD1012 (Fig. 4A, lane 2) and was similar to the level of SOS mutagenesis observed for both umuD+ and umuD1012C in a recA352 (coprotease-deficient mutant) genetic background (Fig. 4C). In contrast to the recA+ genetic background, umuD1012C was proficient in SOS mutagenesis in both the recA441 and recA730 genetic backgrounds (Fig. 4D and E). This activity correlates with the abilities of these recA gene products to facilitate the self-cleavage of UmuD1012 (Fig. 4A, lanes 9 and 10 and lanes 13 and 14), a prerequisite for activation of UmuC as a lesion bypass DNA polymerase (43, 55), and is consistent with the comparatively robust activity of umuD1012C+ in SOS mutagenesis as measured by the arginine reversion assay (Fig. 3). Taken together, these results confirm that umud1012C+ is active in SOS mutagenesis but umud1012C+ is not and further support the notion that Leu101 and Arg102 of UmuD are critical for the ability of these recA gene products to facilitate the self-cleavage of UmuD to yield UmuD+ but, subsequent to cleavage, are of only moderate importance for the role of UmuD+ in TLS.

UmuD1012 can undergo RecA-ssDNA-facilitated cleavage in vitro. To follow up on our observation that wild-type RecA protein could not facilitate efficient cleavage of UmuD1012 in vivo, we investigated whether highly purified UmuD1012 would be cleaved when incubated with purified RecA protein and ssDNA in vitro. In our first experiment, we investigated the efficiency of self-cleavage of UmuD1012 relative to that of wild-type UmuD as a function of time using a fixed concentration of RecA protein (4.6 μM as a monomer) and ssDNA (1.7 μM). Under these conditions, the ssDNA was slightly more than twofold in excess of that necessary for complete nucleation of RecA (~6 RecA molecules can bind to a single 20-mer ssDNA) (28). In striking contrast to our expectations based on in vivo observations, we did observe rather efficient cleavage of UmuD1012 to yield UmuD+ in vitro (Fig. 5A). Densitometric analysis of the Coomassie blue-stained gel relative to appropriate standard curves indicated that although UmuD1012 was activated for cleavage by RecA-ssDNA in vitro, it nevertheless underwent self-cleavage less efficiently than did wild-type UmuD under the identical conditions (Fig. 5B).

To further characterize the ability of UmuD1012 to be cleaved by RecA-ssDNA in vitro, we investigated the efficiency of UmuD1012 cleavage relative to that of wild-type UmuD as a function of the RecA concentration over a 16-fold range (from 0.58 to 9.2 μM) using a fixed level of ssDNA (1.7 μM) and a fixed time of incubation. Whereas the efficiency of wild-type UmuD cleavage mediated by RecA and ssDNA appeared to be directly proportional to the amount of RecA protein added starting at molar ratios of UmuD2 (as a dimer) to RecA (as a monomer) in panel A was 1:1.4. The molar ratios of UmuD2 (as a dimer) to RecA (as a monomer) in panel C are indicated (UmuD2/RecA). Densitometric analysis (B and D) of the results shown in panels A and C was performed using the Molecular Analyst software package (Bio-Rad).
age of UmuD1012 was observed only at ratios of UmuD1012 to RecA between 1:0.69 and 1:1.4 (Fig. 5C). Furthermore, regardless of the concentration of RecA protein investigated, cleavage of UmuD1012 was significantly less efficient than that observed for wild-type UmuD (Fig. 5D), consistent with the results of the time course experiment (Fig. 5B).

Our inability to detect cleavage of UmuD1012 in vivo in a recA+ genetic background (Fig. 4A) contrasts with the rather efficient cleavage observed in vitro with a reconstituted self-cleavage assay comprising purified proteins (Fig. 5A and B). We have previously observed rather striking differences between cleavage of certain mutant UmuD proteins in vivo versus their cleavage in vitro under conditions similar to those used here (19, 31). Our inability to observe cleavage of UmuD1012 by wild-type RecA in vivo is presumably attributable to the numerous protein-protein interactions that occur in vivo between UmuD2, as well as RecA, and other proteins that are absent from our in vitro system, including RecA-ssDNA-facilitated cleavage of LexA (35) and RecA-ssDNA-dependent homologous recombination (54), both of which represent processes that compete directly with UmuD cleavage.

Finally, a rough estimation of the steady-state levels of UmuD, UmuD1012, and RecA under the conditions of our in vivo cleavage assay provides further support for this conclusion (data not shown). When UmuD and UmuD1012 were expressed from multicopy plasmids in vivo to measure cleavage efficiency, the approximate molar ratio was 1 molecule of UmuD2 (as a dimer) to 1.8 to 3.8 molecules of RecA (as a monomer). The molar ratio of UmuD2 to RecA (and UmuD1012 to RecA) analyzed in our in vitro system varied from a low of 1:2.8 to a high of 1:0.18 (Fig. 5C). Given that only a portion of the total RecA protein in vivo is likely to be available to facilitate UmuD cleavage (see above) and that cleavage of UmuD1012 in vitro requires higher levels of RecA than those required by wild-type UmuD (i.e., UmuD1012/RecA ratios on the order of 1:0.69 to 1.4 [or more] [Fig. 5C and D]), we suggest that UmuD1012 is not cleaved to an easily detectable level in vivo because of limiting levels of RecA-ssDNA nucleoprotein filaments and not because of widespread differences between our in vitro and in vivo systems with respect to their underlying mechanisms.

**UmuD1012 can interact directly with the RecA-ssDNA nucleoprotein filament in vitro.** Cleavage of purified UmuD1012 in vitro was very sensitive to the concentration of RecA protein (Fig. 5C and D), suggesting that UmuD1012 might have a reduced affinity for the RecA-ssDNA nucleoprotein filament. To test whether UmuD1012 was affected in interaction with RecA, we employed a cross-linking assay developed by Frank et al. (14). Under the conditions used in this analysis, both UmuD and UmuD1012 were essentially comparable with respect to their abilities to be cross-linked to RecA-ssDNA nucleoprotein filaments (Fig. 6). Our finding that UmuD1012 was able to interact with the RecA-ssDNA nucleoprotein filament in a manner grossly similar to that of wild-type UmuD is consistent with our finding that purified UmuD1012 can be cleaved by RecA and ssDNA in vitro.

**UmuD1012 is defective in inhibition of RecA-ssDNA-facilitated homologous recombination.** Although UmuD’1012 was active in SOS mutagenesis (Fig. 3), it was only ~60% as active as wild-type UmuD’, suggesting that Leu101 and Arg102 might contribute in some relatively modest way towards TLS. Another biological property of UmuD’ and UmuC is their ability to inhibit RecA-mediated homologous recombination (53, 59, 63). In vivo, this inhibition requires that their expression be elevated to higher than physiological levels (3). Sommer and coworkers have proposed that this antirecombination activity of UmuD’ together with UmuC is physiologically relevant and important for regulating the activity of RecA protein such that homologous recombination can be attenuated to permit TLS once UmuD’ and UmuC have accumulated to sufficient levels (59).

To investigate whether Leu101 and Arg102 are important for the antirecombination activity of UmuD’C, we measured the effects of \textit{umuD1012C} and \textit{umuD’1012C} on homologous recombination relative to those of the wild-type operons. This was done by quantitating the respective efficiencies of P1vir-mediated transduction of the \textit{crcA280:Tn10} locus as a function of the various plasmid-carried \textit{umuDC} operons. \textit{crcA} is an unessential gene that confers camphor resistance (2). The host strain contained a deletion of the chromosomal \textit{umuDC} locus and expressed elevated levels of the various plasmid-encoded Umu proteins by virtue of a \textit{lexA}(Def) mutation. With this approach, we observed ~4.3- and ~21-fold inhibition of homologous recombination by wild-type \textit{umuDC} and \textit{umuDC’}, respectively, relative to that observed for the pBR322kan control (Fig. 7). Interestingly, whereas \textit{umuD1012C} was only ~2-fold less efficient at inhibiting homologous recombination than was \textit{umuD’C}, \textit{umuD’1012C} was ~13.5-fold less efficient than was \textit{umuD’C} (Fig. 7), thus indicating that Leu101 and Arg102 of UmuD’ are important for its antirecombination activity. Consequently, although the inability of \textit{umuD’1012C} to effectively antagonize homologous recombination might account for its modest defect in TLS (Fig. 3), the fact that it was ~60% as active as wild-type \textit{umuD’C} in SOS mutagenesis suggests that this antagonistic activity is not a strict requirement for TLS in vivo under our experimental conditions.
DISCUSSION

Model for RecA-ssDNA-facilitated cleavage of UmuD. In this report we describe our genetic and biochemical characterizations of the umuD1012 and umuD’1012 alleles, each encoding glycines in place of Leu101 and Arg102. Our findings that UmuD1012 undergoes RecA-ssDNA-facilitated cleavage less efficiently than wild-type UmuD, both in vivo and in vitro, and that umuD’1012C’ is severely impaired in inhibition of RecA-ssDNA-mediated homologous recombination in vivo indicate that Leu101 and Arg102 are important for interaction of the umuD gene products with RecA-ssDNA nucleoprotein filaments. In contrast, Leu101 and Arg102 are of only modest importance for TLS in vivo; umuD’1012C’ was ~60% as proficient as wild-type umuD’C’ in SOS mutagenesis.

Our finding that UmuD1012 was not grossly affected in interaction with RecA-ssDNA nucleoprotein filaments in vitro, as measured by solution cross-linking, suggests that the deficiency of UmuD1012 in cleavage by RecA is due to a reduced ability of UmuD1012 to undergo the presumably subtle RecA-induced conformational change that leads to UmuD cleavage. In this respect, it is interesting that Leu101 and Arg102 are necessarily only a few residues away from Lys97, which is critical for UmuD cleavage (Fig. 8A). In the Lys-Ser dyad found both in the signal peptidases (48, 49) and in UmuD and related molecules (43), the exact position of the Lys clearly must be crucial for activating the Ser (Ser60 in UmuD) to act as a nucleophile in the cleavage reaction (33, 34). Thus, a plausible model to explain our results is that Leu101 and Arg102 interact with the RecA-ssDNA nucleoprotein filament in such a way that the loop containing Lys97 is pushed, thereby bringing Lys97 closer to Ser60 (Fig. 8B and C). This, together with numerous other contacts between the RecA-ssDNA nucleoprotein filament and both the amino-terminal arm (19, 31) and the carboxy-terminal globular domain of UmuD (32), is presumably sufficient for the proper alignment of the Ser60-Lys97 catalytic dyad with the cleavage site (located between residues 24 and 25) for activation of the intrinsic protease activity of UmuD. Consistent with this model, the recently solved solution structure of the UmuD2 homodimer (Ferentz et al., unpublished data) indicates that in UmuD’2, the terminal group of Lys97 is not as ideally positioned to deprotonate Ser60 as suggested by the crystal (50) but rather is farther away from Ser60 and would thus require an external force to push them together.

The UmuD-like mutagenesis proteins and the LexA-like transcriptional repressors utilize nonidentical sets of contacts with RecA-ssDNA to facilitate their cleavage. Our finding that Leu101 and Arg102 are critical for cleavage of UmuD yet are not conserved among the structurally related transcriptional repressors that also undergo RecA-ssDNA-facilitated cleavage (1, 17) raises the question of why the UmuD-like and LexA-like subfamilies employ nonidentical sets of contacts to enable self-cleavage. Three possible explanations for this difference are as follows: (i) the UmuD-like proteins have a different and smaller amino-terminal domain than do the transcriptional repressors (52) and therefore require a different set of contacts with the RecA-ssDNA nucleoprotein filament to facilitate their cleavage; (ii) UmuD-like proteins undergo physical interactions with partner proteins (i.e., UmuC and its homologs) (4, 22, 70), as well as with other proteins involved in recombination (5, 32) and replication (62), that collectively demand that the UmuD-like proteins undergo interaction with RecA-ssDNA nucleoprotein filaments via a different set of contacts; and (iii) the use of a nonidentical set of contacts to differen-
temporarily activate cleavage constitutes a device whereby the cell is able to achieve radically distinct cleavage kinetics for LexA relative to UmuD.

Given that LexA protein represses the SOS regulon while UmuD, although inactive in TLS (43), appears to act as part of a primitive cell cycle checkpoint control (41, 46), it seems reasonable that the cleavage kinetics of LexA and UmuD would differ. Whereas rapid cleavage of LexA in response to DNA damage is desirable for the timely derepression of the SOS-regulated gene products, the comparatively slow cleavage of UmuD presumably allows additional time for the repair of damaged DNA by accurate repair mechanisms such as nucleotide excision repair (46). Consequently, the timed delay in cleavage of UmuD to yield UmuD\textsuperscript{9} may have been optimized through evolution, in part through the use of a different set of contacts between UmuD and RecA-ssDNA nucleoprotein filaments, to allow maximum cell survival, first via a UmuD\textsubscript{C}-dependent checkpoint control (41, 46) and second via UmuD\textsubscript{C}-dependent TLS (17). The finding by McDonald et al. that this comparatively low rate of UmuD self-cleavage is due primarily to its being a poor substrate and not to its being a poor enzyme (37, 38) is consistent with this model.

Our results, indicating that the UmuD-like mutagenesis proteins and the LexA-like transcriptional repressors utilize non-identical sets of contacts with the RecA-ssDNA nucleoprotein filaments to promote self-cleavage, are consistent with earlier reports that RecA is similarly thought to use unique sets of contacts with the various repressors and UmuD to facilitate their cleavage. Four recA alleles, recA\textsubscript{91} (G229S), recA\textsubscript{430} (G204S), recA\textsubscript{1730} (S117F), and recA\textsubscript{1734} (R243L), exhibit differential coprotease activities towards the lacI repressor, the \(\phi\)80 repressor, LexA, and UmuD (reviewed in reference 28).

Although the RecA\textsubscript{91} protein can promote the cleavage of the lacI repressor but not the \(\phi\)80 repressor (8, 44), the RecA\textsubscript{430} protein can promote the cleavage of the \(\phi\)80 repressor but not the lacI repressor, LexA, or UmuD (5, 7). By contrast, the RecA\textsubscript{1730} protein can promote cleavage of the lacI repressor, the \(\phi\)80 repressor, and UmuD but not LexA (8). Finally, the RecA\textsubscript{1734} protein can promote the cleavage of the lacI repressor and LexA but not the \(\phi\)80 repressor or UmuD (8). Results similar to these have also been found with respect to differences in cleavage of UmuD and LexA in vivo by plasmid-carried recA alleles with P67D, P67R, E154D, or E154Q substitutions (27, 42).

**Relationship between the roles of the umuD\textsubscript{C} gene products in SOS mutagenesis and in inhibition of RecA-mediated homologous recombination.** In addition to its role in TLS, elevated levels of UmuD\textsuperscript{D} together with UmuC act to antagonize RecA-mediated homologous recombination in vivo (59). It has been suggested that this inhibition of homologous recombination from Ser60 (Ferentz et al., unpublished data). In the model (C), RecA-ssDNA pushes on Leu101-Arg102, leading to the repositioning of Lys97 relative to Ser60. This repositioning of Lys97, together with numerous other contacts between UmuD and RecA-ssDNA (19, 31, 32), leads to the activation of Ser60 and subsequent cleavage of UmuD to yield UmuD\textsuperscript{D}. For simplicity, the cartoons depict the catalytic dyad and Leu101-Arg102 of a single UmuD protomer; its intradimer partner and the amino-terminal arms containing the cleavage sites are not shown. See the text for further details.
tion might result from an effect on the formation of RecA-ssDNA filaments and may constitute a general mechanism by which RecA-mediated homologous recombination is attenuated to allow TLS (59, 63). Our finding that umuC Δ102 is able to efficiently promote SOS mutagenesis, despite its inability to efficiently inhibit RecA-mediated homologous recombination in vivo, indicates that this inhibition of recombination is not a prerequisite for SOS mutagenesis under our experimental conditions. This interpretation is consistent with the finding that a recA mutation with an N113K change was refractory to the inhibitory effect of elevated levels of UmuD′ C but was nonetheless proficient in SOS mutagenesis (60). Although other recA alleles shown to be similarly refractory to the inhibitory effect of elevated levels of UmuD′ C were less active in SOS mutagenesis (60), it is possible that their defect in SOS mutagenesis was due to a deficiency of the mutant RecA proteins in TLS rather than to their reduced sensitivity to UmuD′ C.

Recent electron microscopy studies have suggested that UmuD′ C binds preferentially to the tip of the RecA-ssDNA filament but at higher levels can also bind within the helical groove of the filament (13). Based on this and other findings (53, 59, 60, 63), it has been suggested that the binding of UmuD′ C to the helical groove might competitively inhibit RecA-mediated homologous recombination, while its interaction with the tip of the RecA-ssDNA filament might deliver UmuD′ C to the site of the lesion. Further work will be required to see whether the UmuD′102 protein is affected in its interaction with the groove and/or the tip of RecA-ssDNA nucleoprotein filaments. These and related studies may add additional insights into the molecular mechanism of RecA-ssDNA-mediated self-cleavage of UmuD2, thus allowing us to test further our model for the role(s) of Leu101 and Arg102 in self-cleavage (Fig. 8).

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