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Intermolecular cleavage by UmuD-like mutagenesis proteins

(MucA/self-processing reactions/protein–protein interactions)

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ABSTRACT **The activity of a number of proteins is regulated by self-processing reactions. Elegant examples are the cleavage of the prokaryotic LexA and λCI transcriptional repressors and the UmuD-like mutagenesis proteins. Various studies support the hypothesis that LexA and λCI cleavage reactions are predominantly intramolecular in nature. The recently described crystal structure of the *Escherichia coli* UmuD′ protein (the posttranslational cleavage product of the UmuD protein) suggests, however, that the region of the protein corresponding to the cleavage site is at least 50 Å away from the catalytic active site. We considered the possibility, therefore, that the UmuD-like proteins might undergo self-processing that, in contrast to LexA and λCI, occurs via an intermolecular rather than intramolecular reaction. To test this hypothesis, we introduced into *E. coli* compatible plasmids with mutations at either the cleavage or the catalytic site of three UmuD-like proteins. Cleavage of these proteins only occurs in the presence of both plasmids, indicating that the reaction is indeed intermolecular in nature. Furthermore, this intermolecular reaction is completely dependent upon the multifunctional RecA protein and leads to the restoration of cellular mutagenesis in nonmutable *E. coli* strains. Intermolecular cleavage of a biotinylated UmuD active site mutant was also observed *in vitro* in the presence of the wild-type UmuD′ protein, indicating that in addition to the intact UmuD protein, the normal cleavage product (UmuD′) can also act as a classical enzyme.**

Since the discovery that the *Escherichia coli* transcriptional repressor LexA possesses the ability to facilitate its own cleavage (1), it has served as a paradigm for studies on self-cleaving reactions (2). Autodigestion of LexA occurs at alkaline pH and is independent of protein concentration, indicating that this autodigestion is an intramolecular reaction (3). Similarly, mutants of the λCI repressor that inefficiently dimerize actually undergo autodigestion at a faster rate than the wild-type λCI, suggesting that this cleavage also occurs via an intramolecular reaction (3, 4). By using a number of hypercleavable (Ind^S) LexA mutants, as well as N-terminal and C-terminal truncated proteins, Kim and Little (5) were, however, able to demonstrate that under certain alkaline conditions *in vitro,* LexA and λCI proteins could act as true enzymes by performing intermolecular cleavage.

The *E. coli* UmuD protein undergoes a self-cleavage reaction that generates UmuD′, a functionally active protein that with UmuC, RecA, and DNA polymerase III facilitates error-prone translesion synthesis of DNA opposite a damaged template DNA (6, 7). Interestingly, UmuD and a family of naturally occurring UmuD-like mutagenesis proteins are roughly the same size as and share limited structural homology with the recombinant C-terminal LexA and λCI enzymes used by Kim and Little (5), suggesting that they too might possess true enzymatic activity. The crystal structure of UmuD′ reveals that the catalytic active site consists of conserved serine and lysine residues that are found at the end of a cleft in the protein, and the cleavage reaction appears to be akin to that by which the TEM-1 β-lactamases inactivate their substrates (e.g., penicillin) (8). Although the N-terminal tail of UmuD′ (corresponding to the cleavage site in UmuD) is disordered, regions of structured protein close to the cleavage site are more than 50 Å away from the active site. On the basis of the structure of UmuD′ alone, one would have to hypothesize that the N-terminal tail of UmuD would have to adopt a significantly different structure (at least around the cleavage site) if it were to undergo an intramolecular self-cleavage reaction (8). Another possibility is, however, that unlike the related LexA and λCI proteins, the UmuD self-processing reaction could predominantly occur via an intermolecular rather than an intramolecular mechanism. To test such an hypothesis, we have generated plasmid-encoded mutations at the cleavage site and active site of several UmuD-like proteins. These plasmids were subsequently introduced, alone or together, into *E. coli* strains normally proficient for cleavage. Interestingly, cleavage only occurs when both plasmids are present. Such observations therefore support the hypothesis that cleavage of the UmuD-like proteins can occur via an intermolecular pathway.

MATERIALS AND METHODS

Generation of Cleavage Site and Active Site Mutants. Specific mutations in either the catalytic active site (active site mutant, ASM) or at the cleavage site (cleavage site mutant, CSM) were generated by using the Stratagene Quick Change mutagenesis kit and by following the manufacturer's suggested protocols (see below for PCR primer sequences). ASM mutations were generated in medium- or high-copy-number plasmids, whereas CSM mutations were generated in low-copy-number plasmids (Fig. 1). Because the medium- or high-copy-number plasmids are compatible with the low-copy-number plasmids, both the ASM and CSM mutants can be expressed together in *E. coli*. In addition, because the CSM mutations were generated in low-copy-number plasmids containing the native *umu* or *muc* promoters, the CSM proteins are only produced at 3- to 5-fold higher levels than those expressed chromosomally.

In our initial attempts to generate a CSM, we found that although changing one of the cleavage site residues from Gly to Asp or to Ser dramatically reduced the extent of cleavage, significant *in vivo* cleavage was still detected with the *E. coli* and *Salmonella typhimurium* UmuD proteins. As a conse-

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Abbreviations: ASM, active site mutant; CSM, cleavage site mutant; MMS, methyl methanesulfonate.

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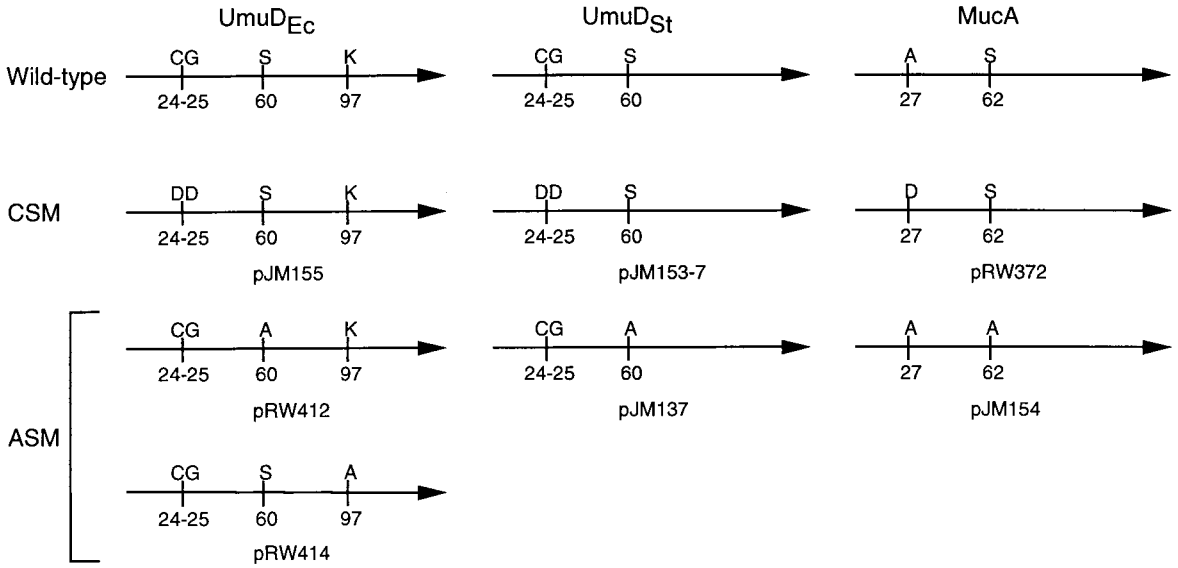

FIG. 1. CSM and ASM of *umuD* and *mucA*. Noncleavable *umuD* mutations and noncleavable *mucA* mutations were generated by using the Stratagene Quick Change mutagenesis kit by following the manufacturer's suggested conditions (Stratagene). The CSM from *E. coli,* and *S. typhimurium umuD* and *mucA* were generated in, or cloned into, pGB2 derivatives (12) (low-copy-number plasmid; three to five copies per cell) and are expressed from the wild-type *umu* or *muc* promoters. Therefore, all three CSM plasmids should express mutant proteins at or near to chromosomally encoded levels. Plasmid pJM155 also expresses UmuC, while pRW372 expresses MucB. The ASMs were generated in, or cloned into, compatible medium- or high-copy-number plasmids and are also expressed from their wild-type promoters. In contrast to the MucA ASM plasmid, which also coexpresses MucB, the *E. coli* and *S. typhimurium* ASM plasmids only express the mutant UmuD protein.

quence, we were forced to generate CSMs in which both residues of the Cys-Gly cleavage site were changed to Asp-Asp (Fig. 1). In contrast, a single change of Gly to Asp at the cleavage site in MucA appeared to render it noncleavable (9, 10). ASMs were constructed that changed the conserved Ser or Lys residues to Ala (Fig. 1).

The double CSM (C24D and G25D) was incorporated into the *E. coli umuD* gene in pRW154 (26), to generate pJM155 by using primers CDGDA (5′-GACCTTGTTCAGGATGACTTTCCGTCACCGGCAG-3′) and CDGCB (5′-CGGTGACGGAAAGTCATCCTGAACAAGGTCGCTA-3′). For ease of subsequent identification, these primers introduce two new silent restriction sites, *Eco*NI and *Ahd*I, into the *E. coli umuD* CSM. The same double CSM (C24D and G25D) was incorporated into the *S. typhimurium umuD* gene in pJM138, a low-copy-number pGB2 (12) derivative that only expresses UmuD and not UmuC, to generate pJM153–7 by using primers CDGDSA (5′-TTAGTTCCGGATGACTTCCCGTCCCCCGCGGCG-3′) and CDGDSB (5′-GGGGGACGGGAAGTCATCCGGAACTAAGTAACT-3′). These primers also create two silent restriction sites, *Bsp*EI and *Ahd*I. In a similar manner, Ser-60 of *S. typhimurium umuD* was changed to Ala (ASM) by using primers SA60SC (5′-TTCAATCATTGCATCTCCGGAGGCTTTGACAAA-3′) and SA60SD (5′-AAAGCCTCCGGAGATGCAATGATTGAAGCAGGC-3′) (which creates a new silent restriction site, *Bsp*EI) to generate pJM137. The Ser-62 residue of *mucA* in pRW72 (10) was changed to Ala (ASM) using primers SA62MA (5′-GTTTCTGGCAGCGCTATGGAAGATGGCCGCATC-3′) and SA62MB (5′-GCCATCTTCCATAGCGCTGCCAGAAACCCGCAG-3′) (which creates a new silent restriction site, *Eco*47III) to generate pJM154. The *E. coli umuD*S60A ASM (pRW412) and the *umuD*K97A ASM (pRW414) were made from plasmids pGW2112 and pGW2115, respectively (11), and were generated by *Hin*dIII digestion and subsequent recircularization of the parental vector fragment. The low-copy-number *mucA*G27D CSM plasmid (pRW372) was generated by cloning an ~2-kb *Xho*I fragment from pTS612 (9) into the *Sal*I site of pGB2 (12). All ASM and CSM plasmids generated via PCR were sequenced to ensure that no gratuitous mutations were incorporated into *umuD* or *mucA*.

Monitoring Intermolecular Cleavage *in Vivo*. Cultures were grown and protein extracts made as described (13). The cleavage reaction was monitored by Western blot analysis using affinity-purified anti-UmuD (14) or anti-MucA (10) antibodies. In these experiments, strains harbor the ASM, the CSM, or both ASM and CSM UmuD or MucA plasmids. For strains that carry coprotease constitutive *recA* alleles (*recA730*/*recA718*), overnight cultures were simply diluted 1:100 into fresh LB medium and grown with shaking at 37°C for ~3 hr until they reached an OD_{600} of 0.6–0.8. At which time, cells were harvested by centrifugation and the resulting cell pellet was resuspended in electrophoresis sample buffer. Cells were lysed by repeated freeze–thaw cycles and the resulting extract was subjected to SDS/PAGE in 15% gels. Proteins were electrotransferred to an Immobilon P membrane (Millipore) and subsequently probed with a 1:10,000 dilution of affinity-purified polyclonal antiserum raised against UmuD/UmuD′ or a 1:5,000 dilution of polyclonal antiserum raised against MucA. The UmuD/D′ and MucA/A′ proteins were subsequently visualized by using the disodium3-[4-methoxyspiro(1,2-dioxetane-3,2′-(5′chloro)tricyclo-[3.3.1.1.^{3,7}]decan)-4-yl]phenylphosphate (CSPD) Western light chemiluminescent assay (Tropix, Bedford, MA).

For strains carrying either a *recA*$^+$ or Δ*recA* allele, the protocol was very similar except that overnight cultures were diluted 1:100 into two duplicate tubes. After ~1.5 hr, mitomycin C (2 μg/ml for UmuD-plasmid-containing strains and 5 μg/ml for MucA-plasmid-containing strains) was added to one of the tubes and growth continued for an additional 2 hr. At that time, both the untreated and mitomycin C-treated cells were harvested and processed as noted above.

***In Vitro* Cleavage of Biotinylated *E. coli* UmuDK97A Protein.** The *E. coli umuD*K97A mutant was subcloned from pGW2115 as a *Cla*I–*Hin*dIII fragment into the similarly digested vector pJM103 [a derivative of pET22b (Novagen)]. This new plasmid, termed pEC69, was then transformed into strain RW382 [a Δ(*umuDC*)*595*::*cat* derivative of BL21(DE3)].

The UmuDK97A protein was subsequently overproduced and purified to homogeneity by using the same protocol as described for the wild-type UmuD protein (15). The purified protein was biotinylated as described (16) by using E2-link Sulfo-NHS-biotin (Pierce) and used as a substrate for the *in vitro* cleavage reaction. The RecA–DNA–UmuD′ complex was formed in two steps: first by making a RecA–DNA nucleoprotein complex in a 2.5-μl reaction mixture containing 10 ng of single-stranded φX174 DNA (New England Biolabs), 0.8 μg of RecA protein (New England Biolabs), 20 mM Tris·HCl (pH 7.5), 15 mM NaCl, 1 mM DTT, 1 mM adenosine 5′-[γ-thio]triphosphate, 1 mM $MgCl_2$, and 0.1 mM EDTA. After a 30-min incubation at 37°C, various amounts of UmuD′ from 0 to 1 μg were added to the preformed RecA–DNA complex and incubated for another 30 min at 37°C. After this time, the newly formed RecA–DNA–UmuD′ complex was mixed with 0.08 μg of the biotinylated ASM UmuDK97A protein and 0.1 μg of BSA, and the reaction volume adjusted to a total of 10 μl. This final nucleoprotein–enzyme complex was subsequently incubated at room temperature for 15 min. Proteins were separated by SDS/PAGE and cleavage of the biotinylated ASM protein was detected by the chemiluminescent assay described above except that streptavidin-alkaline phosphatase-conjugated antibodies (Tropix) were used instead of the primary anti-UmuD and secondary goat anti-rabbit alkaline phosphatase antibodies.

Mutagenesis Assays. Mutagenesis assays were performed as described (17). Bacterial cultures were grown overnight in LB medium containing the appropriate antibiotics. Aliquots (1 ml) were centrifuged and resuspended in an equal volume of SM buffer (18). The ability of particular plasmid bearing strains to promote Umu-dependent SOS mutator activity in the absence of exogenous DNA damage was judged by plating 100-μl aliquots on Davis and Mingioli minimal agar plates supplemented with a trace amount of histidine (1 μg/ml) (19). Chemically induced mutagenesis was determined as described above, except that 5 μl of a 1:5 dilution of methyl methanesulfonate (MMS, Sigma) in dimethyl sulfoxide (Sigma) was applied to a small sterile disk in the center of the plate. Both spontaneously arising and MMS-induced His^+ mutants were scored after 4 days of incubation at 37°C. The results represent the average number of His^+ colonies from at least three cultures from each strain, with eight plates per culture.

RESULTS

Intermolecular Cleavage of UmuD-Like Proteins. To test the hypothesis that UmuD-like proteins are capable of cleavage via an intermolecular reaction, we generated ASM and CSM mutants in three related proteins: the *E. coli* UmuD protein, the *S. typhimurium* UmuD protein, and the R46/pKM101-encoded MucA protein (Fig. 1). We hypothesized that if the UmuD-like proteins undergo a self-processing reaction that is intermolecular in nature, then neither the ASM nor the CSM monomers would be cleaved individually but that if introduced together into the same cell, the CSM, which still retains a functional active site, could act as an enzyme to promote cleavage of the ASM, which still retains a functional cleavage site.

When either the CSM or ASM was introduced individually into an *E. coli* strain carrying a Δ*umuDC* mutation and the *recA730* mutation (which normally results in constitutive coprotease activity toward UmuD) (13, 20), no detectable cleavage of UmuD or MucA was observed (Fig. 2). In contrast, however, when both plasmids (CSM and ASM) were introduced together into the same strain, cleavage products of UmuD′ or MucA′ were clearly detectable. Moreover, because neither protein can autodigest via an intramolecular reaction, this observation supports the hypothesis that cleavage can occur via an intermolecular reaction *in vivo*. Indeed, this appears to be a true enzymatic reaction because the active enzyme (CSM) was cloned into a low-copy-number plasmid and the substrate (ASM) was cloned into a medium-copy-number vector. Furthermore, these reactions all occurred with compatible ASM and CSM plasmids encoding the *E. coli* UmuD, *S.typhimurium* UmuD, or MucA proteins, indicating that the intermolecular cleavage reaction is ubiquitous among the UmuD-like mutagenesis proteins (Fig. 2).

Intermolecular Cleavage of the UmuD-Like Proteins Requires RecA. It has previously been shown that under physiological conditions, RecA acts as an effector to stimulate the latent capacity of this class of protein to autodigest (1). We were therefore interested in determining whether the intermolecular reaction observed above was also dependent upon RecA (Fig. 3). Indeed, by using various strains of *E. coli* that differ in their RecA coprotease activity, we determined that the intermolecular cleavage reaction is absolutely dependent upon functional RecA protein: cells proficient for coprotease activity (*recA730*/*recA718*) yielded significant levels of the UmuD′ product (Fig. 2 and see Fig. 5), whereas wild-type $recA^+$ cells did so only after exposure to DNA damaging agents (Fig. 3). In contrast, cells carrying a Δ*recA* mutation were unable to perform the intermolecular reaction even after DNA damage (Fig. 3). In light of these findings, the intermolecular reaction should, perhaps, be considered trimolecular because in addition to two molecules of UmuD (or MucA), it also requires RecA. In the past, it has been assumed that the coprotease activity of RecA provides a localized environment in which the Lys-97 residue of the UmuD active site is deprotonated (21). Although this may be true, we propose that RecA also acts as a molecular chaperone (22) by providing a scaffold on which the two UmuD (or MucA) protomers are

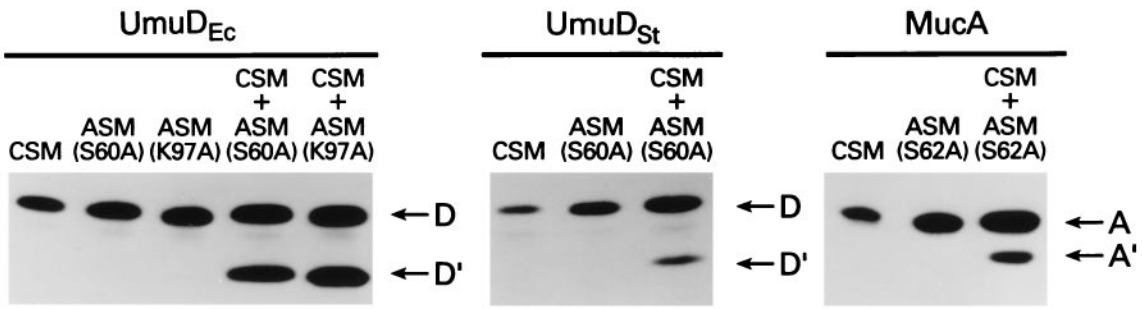

FIG. 2. Intermolecular cleavage of UmuD and MucA *in vivo*. Whole-cell extracts were made from the *E. coli* strain, RW244 [relevant genotype: *recA730*, *lexA51*(Def), Δ(*umuDC*)*595*::*cat*] containing plasmids encoding the mutant *umuD* and *mucA* genes, either singly or doubly (ASM plus CSM). The SOS regulon is fully derepressed in RW244 due to a defective LexA protein, and therefore, the UmuD and MucA proteins should be constitutively expressed. In addition the RecA730 protein is constitutively activated for coprotease functions and as a consequence, the wild-type UmuD and MucA proteins are efficiently converted to their respective cleavage products, UmuD′ and MucA′ in the absence of exogenous DNA damage (10, 13).

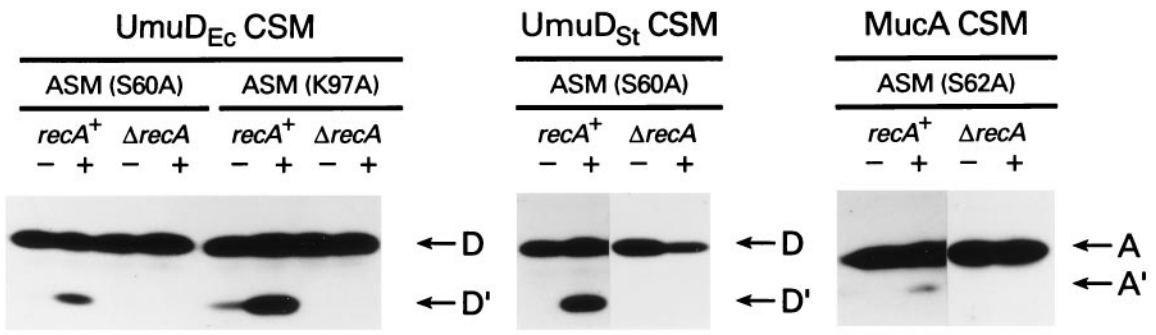

FIG. 3. *In vivo* intermolecular cleavage of UmuD requires RecA. To demonstrate that the intermolecular cleavage is dependent on RecA, protein extracts were made from either strain RW218, a wild-type *E. coli* strain [relevant genotype: *recA*$^+$ *lexA71*(Def), Δ(*umuDC*)*595*::*cat*] or RW174 (10), a *recA* deletion strain [relevant genotype: Δ*recA*, *lexA51*(Def), Δ(*umuDC*)*595*::*cat*]. Lanes: +, extracts from cells exposed to mitomycin C; −, extracts from untreated cells.

correctly aligned. As a consequence, the cleavage site and the active site of both protomers appropriately interact with each other such that efficient intermolecular cleavage occurs.

Intermolecular Cleavage of *E. coli* UmuD *in Vitro*. To demonstrate that the intermolecular reaction also occurs *in vitro*, we have purified the K97A mutant UmuD protein to homogeneity. The purified K97A mutant protein was subsequently biotinylated and cleavage was monitored by probing with streptavidin-alkaline phosphatase-conjugated antibodies followed by chemiluminescent detection. Under these conditions, we were able to determine whether the intermolecular reaction could occur *in vitro* in the presence of the wild-type UmuD or UmuD′ proteins. Indeed, these *in vitro* studies revealed that both the intact UmuD (data not shown) and processed UmuD′ proteins can act as enzymes in the RecA-mediated reaction *in vitro* (Fig. 4). The efficiency of the reaction was, in general, somewhat lower than that observed *in vivo,* suggesting that additional factors necessary for the efficient intermolecular cleavage *in vivo* are absent in the *in vitro* reactions.

Restoration of Mutagenesis Functions. Because of an inability to undergo the self-processing reaction and thereby generate the mutagenically active UmuD′ protein, Δ*umuDC* cells carrying either the CSM or ASM plasmids alone are rendered phenotypically nonmutable (Fig. 5). Because significant accumulation of the mutagenically active UmuD′ protein occurs under conditions where intermolecular cleavage is favored, we hypothesized that this should also lead to the restoration of mutagenesis functions. To test this hypothesis, the ASM and CSM *E. coli* UmuD plasmids were introduced separately or together into a Δ*umuDC* strain that carries another coprotease proficient *recA* mutation, *recA718* (23, 24). *recA718* differs from *recA730*, however, in that the coprotease activity is not expressed unless the SOS regulon is fully derepressed, and even then, coprotease activity is increased when cells are exposed to DNA damaging agents (25). As a consequence of the intermediate level of coprotease activity toward wild-type UmuD protein, *recA718 lexA*(Def) cells exhibit a modest Umu-dependent spontaneous mutator activity (24, 26). By using this strain, we could follow the intermolecular cleavage of UmuD and assay its biological consequences (Fig. 5). Cells carrying either the CSM or ASM plasmids alone were unable to facilitate any self-processing and, as a consequence, the level of Umu-dependent mutagenesis was minimal. In contrast, however, under conditions where the intermolecular cleavage reaction between the ASM (either the S60A or K97A mutation) and the CSM occurred, there were significantly higher levels of cellular mutagenesis. The differences in the level of SOS-dependent spontaneous mutagenesis seen with the two ASM mutants and the wild-type UmuD protein are in accordance with previous studies that demonstrated that under certain conditions, the K97A ASM has a greater effect on the subsequent activity of UmuD′ than the S60A ASM (11).

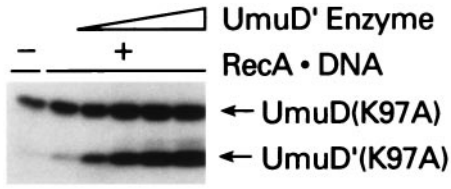

FIG. 4. Cleavage of the biotinylated UmuDK97A ASM mutant protein by a RecA–DNA–UmuD′ complex. Proteins were separated by SDS/PAGE and cleavage of the biotinylated ASM protein was detected by a chemiluminescent assay using streptavidin-alkaline phosphatase-conjugated antibodies. Less, but still significant, cleavage was observed when UmuD was used in the RecA–DNA complex instead of UmuD′ (data not shown). Addition of the RecA–DNA complex (+) is indicated (−, not added). The amount of UmuDK97A (substrate) in the reaction was 0.08 μg. The amount of UmuD′ (enzyme) in the reaction was 0.08 μg, 0.2 μg, 0.5 μg, or 1 μg, respectively (lanes from left to right).

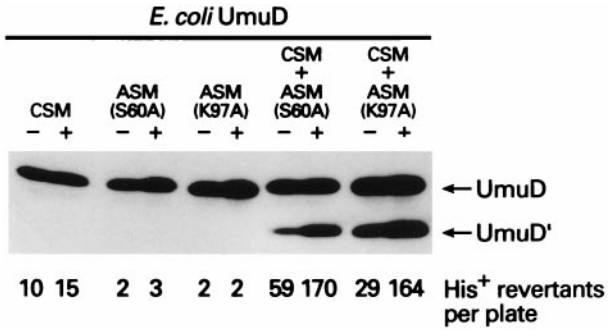

FIG. 5. Intermolecular cleavage of UmuD promotes mutagenesis. Western blot analysis was performed on protein extracts derived from strain RW126 (26) [relevant genotype: *recA718*, *lexA51*(Def), Δ(*umuDC*)*595*::*cat, hisG4*(Oc)] harboring the noncleavable mutant *E. coli* CSM and/or two different ASM plasmids. (Note that the low-copy-number CSM plasmid pJM155, also coexpresses UmuC, but the ASM does not). Culture conditions were as described in Fig. 2. Mutagenesis experiments were performed as described on exactly the same strains used in the Western analysis. The number of His$^+$ revertants per plate represents a mean number from at least three cultures. Lanes : +, extracts from cells exposed to DNA damaging agents (mitomycin C for the Western assay and MMS for the reversion assay); −, extracts from untreated cells. Under these conditions, the control strain RW126 lacking any plasmid gave 2 His$^+$ revertants in the absence of DNA damage and 4 His$^+$ revertants after exposure to MMS. In contrast, RW126/pRW154 (*umuDC* wt) (26) gave 113 His$^+$ revertants in the absence of DNA damage and 430 His$^+$ revertants after exposure to MMS.

DISCUSSION

Intermolecular Cleavage Versus Intramolecular Cleavage. Our results firmly establish that cleavage of UmuD-like proteins can occur via an intermolecular reaction that is reminiscent of more classical intermolecular enzyme–substrate reactions. Our experiments do not, however, address the possibility that UmuD cleavage also occurs via an intramolecular reaction. Indeed, previous experiments have shown that the UmuD-like proteins undergo autodigestion at alkaline pH in the absence of additional cofactors. Because it appears that the intermolecular cleavage reaction is absolutely dependent upon RecA (Fig. 3), it seems reasonable to assume that autodigestion represents cleavage via the intramolecular pathway. On the basis of the rather efficient intermolecular cleavage of *E. coli* UmuD reported herein and its inefficient autodigestion (27), it would appear that *E. coli* UmuD cleavage occurs predominantly via the intermolecular pathway. Such a conclusion may not, however, be true for all UmuD-like proteins. For example, MucA autodigests much more efficiently than *E. coli* UmuD (10) and appears to undergo inefficient intermolecular cleavage (c.f. Figs. 2 and 3, *E. coli* UmuD vs. MucA), suggesting that MucA cleavage predominantly occurs via the intramolecular pathway. Thus, at first glance, there appears to be an inverse relationship between the ability of the proteins to autodigest (via intramolecular cleavage) and their ability to undergo intermolecular cleavage, and it should be interesting to see whether such comparisons can be extended to other proteins that undergo intra/intermolecular cleavage reactions.

Interestingly, it appears that the K97A UmuD ASM is cleaved with somewhat greater efficiency *in vivo* than is the S60A UmuD ASM (Fig. 3). It has previously been shown that the LexA K156A mutant, which is analogous to the K97A UmuD ASM, efficiently inhibits the cleavage of wild-type LexA, thereby suggesting that the LexA K156A mutant binds more tightly to RecA than the wild-type protein (28). Thus, by analogy to the LexA mutant, the K97A UmuD ASM mutant might also be expected to bind RecA more tightly than either wild-type UmuD or the S60A UmuD ASM and, as a consequence, be efficiently cleaved in the intermolecular reaction. In support of this notion, the K97A UmuD ASM even exhibits some cleavage in uninduced cells, which are thought to have only very low levels of activated RecA (Fig. 3).

Protein–Protein Interactions Required for Intermolecular Cleavage. Intermolecular cleavage of UmuD is presumably mediated by protein–protein interactions between a UmuD substrate and a UmuD or UmuD′ enzyme. The crystallized structure (8) and the NMR-derived solution structure (29) of UmuD′ provide clues as to how the substrate and enzyme might associate to facilitate intermolecular cleavage. Such interactions clearly cannot involve residues identified at the crystallized molecular dimer interface because such interactions would occlude the catalytic cleft leading to the active site of the UmuD′ enzyme (8). By comparison, protein–protein interactions involving residues at the crystallized filament dimer interface do not occlude the active site and may, in fact, allow the N-terminal region of one protomer (the UmuD substrate) to interact with the active site of another protomer enzyme (UmuD or UmuD′) (29). Similar to UmuD′, it seems likely that the N-terminal tail of UmuD is somewhat flexible (8, 29) and that the role of the RecA chaperone is to stabilize the transient interactions between the N-terminal tail of a substrate protomer and the active site of an enzyme protomer. Experiments are currently in progress to test this hypothesis that should, hopefully, identify which residues of the substrate protomer and the enzyme protomer are critical for efficient intermolecular cleavage.

Proteolytic Roles for the UmuD-Like and UmuD′-Like Enzymes. Our finding that both the UmuD-like and UmuD′-like proteins can act as enzymes raises the intriguing possibility that these enzymatic activities might be a prerequisite for Umu function. The fact that both the S60A and K97A ASMs, as might be expected, appear to be completely inactivated for proteolytic functions yet are still able to promote significant amounts of cellular mutagenesis (even when expressed in UmuD′; ref. 11 and M. Gonzalez, personal communication) suggests, however, that such activities are not required for SOS mutagenesis. In addition to their extensively characterized roles in damage inducible mutagenesis, the UmuD-like proteins are also believed to play important roles in the ability of *E. coli* cells to recover from the deleterious effects of cellular DNA damage. For example, they appear to help cells restart transiently inhibited chromosomal replication (replication restart) (30) and cell division after DNA damage (31). It is therefore possible that the UmuD-like proteins may act as enzymes in these processes.

Because the UmuD/D′-like proteins contain a catalytic active site and function as enzymes, potential substrates need only contain an appropriate cleavage site. However, as normal cleavage occurs (albeit at different rates) when the actual cleavage site is changed from Cys-Gly (wild type) to Cys-Asp or Cys-Ser (in UmuD) and Ala-Gly (wild type) to Thr-Gly (in MucA) (10) (and perhaps many other combinations), it may be virtually impossible to identify potential substrates based simply on limited structural homology. Clearly, under such situations, the best way to show that a protein is a substrate of the UmuD or UmuD′ enzymes is to physically demonstrate cleavage of the target protein either *in vivo* or *in vitro*.

Finally, our finding that the UmuD-like proteins undergo intermolecular cleavage but contain both a catalytic site and a cleavage site in the same molecule supports the notion that this intriguing state of affairs may reflect an evolutionary intermediate between true self-processing reactions and classical intermolecular enzyme reactions.

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