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Location of Functional Regions of the *Escherichia coli* RecA Protein by DNA Sequence Analysis of RecA Protease-Constitutive Mutants

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In previous work (E. S. Tessman and P. K. Peterson, J. Bacteriol. 163:677–687 and 688–695, 1985), we isolated many novel protease-constitutive (Prt^c) *recA* mutants, i.e., mutants in which the RecA protein was always in the protease state without the usual need for DNA damage to activate it. Most Prt^c mutants were recombinase positive and were designated Prt^c Rec$^+$; only a few Prt^c mutants were recombinase negative, and those were designated Prt^c Rec$^-$. We report changes in DNA sequence of the *recA* gene for several of these mutants. The mutational changes clustered at three regions on the linear RecA polypeptide. Region 1 includes amino acid residues 25 through 39, region 2 includes amino acid residues 157 through 184, and region 3 includes amino acid residues 298 through 301. The in vivo response of these Prt^c mutants to different effectors suggests that the RecA effector-binding sites have been altered. In particular we propose that the mutations may define single-stranded DNA- and nucleoside triphosphate-binding domains of RecA, that polypeptide regions 1 and 3 comprise part of the single-stranded DNA-binding domain, and that polypeptide regions 2 and 3 comprise part of the nucleoside triphosphate-binding domain. The overlapping of single-stranded DNA- and nucleoside triphosphate-binding domains in region 3 can explain previously known complex allosteric effects. Each of four Prt^c Rec$^-$ mutants sequenced was found to contain a single amino acid change, showing that the change of just one amino acid can affect both the protease and recombinase activities and indicating that the functional domains for these two activities of RecA overlap. A *recA* promoter-down mutation was isolated by its ability to suppress the RecA protease activity of one of our strong Prt^c mutants.

The *Escherichia coli* RecA protein has been shown to play a key role in homologous genetic recombination and the induction of the SOS repair system (reviewed in references 17, 19, 29, and 42). In vitro biochemical studies have demonstrated that RecA protein possesses a variety of related enzymatic activities, which include (i) single-stranded DNA (ssDNA)- and double-stranded DNA-dependent ATP hydrolysis (26, 32, 45, 46), (ii) ATP-dependent annealing of homologous ssDNAs (45), (iii) ATP-dependent assimilation of ssDNA into homologous duplex DNA (38), (iv) pairing and exchange of duplex DNA (50), and (v) ATP-dependent and ssDNA-dependent proteolytic cleavage of phage lambda repressor and the LexA protein, the repressor of the SOS genes (5, 16). The *recA* gene has been sequenced; a single polypeptide of molecular weight 37,800 is encoded by 1,059 nucleotides (8, 36).

RecA binds to ssDNA (21, 51) and nucleoside triphosphates (NTPs) (5, 13, 27, 34, 47). The binding of ssDNA and an ATP-like effector to RecA is required for both the recombinase and protease functions of RecA (5, 20, 27, 38, 44). It has been suggested that the same ternary complex of RecA, ssDNA, and ATP is involved in both the activation of RecA protease and the early stages of RecA recombinase function (5, 30, 38, 41) and that the same effector-binding sites on RecA may be used for both functions (30). For elucidation of the role of ssDNA and NTP in promoting RecA function, it should be useful to locate the regions of these two effector-binding sites on the RecA polypeptide.

RecA has been inferred to have two DNA-binding sites (3, 5, 9, 34); one may bind ssDNA, and the other may bind double-stranded DNA (9). It has also been shown that RecA has only one NTP-binding site (5, 13, 46, 47). Identification of the amino acid substitutions in several mutant RecA proteins led Kawashima et al. to propose that the N-terminal region of RecA is involved in ssDNA binding and that Gly-160 participates in ATP binding (10). By using the affinity labels 8-azidoadenosine 5′-triphosphate and 5′-*p*-fluorosulfonylbenzoyladenosine to covalently modify RecA, Knight and McEntee have identified Tyr-264 as the site of modification and have suggested that the polypeptide region around Tyr-264 may be involved in NTP binding (13, 14). It has been predicted that amino acid residues 47 through 250 of the RecA protein would be found to form a core structure that may participate in NTP binding (1). The C-terminal region of RecA may be required for both ssDNA and NTP binding, since a truncated RecA protein that contains 60% of the N-terminal region of RecA can no longer bind ssDNA and ATP (34). The possible role of the C-terminal region of RecA in DNA binding is also indicated by its richness in basic and aromatic amino acids (36).

Under normal growth conditions, the *E. coli* RecA protease is inactive. (Here we retain the term RecA protease for convenience, even though it is not certain whether RecA is a true protease or merely a cofactor for repressor autodigestion [15].) When the DNA of the cell is damaged the RecA protein is activated to the protease state, in which it cleaves a repressor protein, LexA, thereby inducing the synthesis of numerous unlinked genes comprising the SOS system, many of whose products are required for repairing DNA damage (17, 42). In vitro, the activation of RecA protease requires the simultaneous binding to RecA of two effector species, namely, ssDNA and NTP; the latter is preferably dATP or ATP (5, 20, 27, 44). It is likely that any treatment that perturbs the concentration of these two effector species in the cell will affect RecA protease activity (31). DNA-damaging treatments, such as UV irradiation, stimulate RecA protease activity presumably by providing

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ssDNA (17, 27, 30, 31). The *recA441* mutant, a temperature-dependent protease-constitutive *recA* strain, shows strong RecA protease activity at high temperature in the absence of any DNA damage (7). Perturbation of the pool of NTP in *recA441* cells by providing different nucleosides or free bases in the growth medium can affect its RecA protease activity (11, 39, 52). The temperature-dependent constitutive protease activity of the *recA441* strain is enhanced by adding adenine to the growth medium and is inhibited by adding a combination of the nucleosides cytidine plus guanosine (C+G) (11, 39, 52). The inhibitory effect of C+G on RecA441 protease activity can be overcome by adenine; the derivatives of C+G seem to serve as negative effectors, whereas the derivatives of adenine serve as positive effectors, of the RecA protease activity (39). These effectors may compete for the same NTP-binding site on the RecA protein (39).

In vitro, RecA441 protein binds both ssDNA and ATP much more tightly than the wild-type RecA protein and is converted to the protease state by much smaller amounts and shorter fragments of ssDNA (20, 27). It has been proposed that RecA441 is activated by the small single-stranded gaps in the DNA of undamaged cells, perhaps at the replication fork, and thus is presumably altered in the ssDNA-binding site (20, 31). It is possible that some protease-constitutive *recA* mutants, like *recA441*, have an altered ssDNA-binding site.

Various NTP species show different efficiencies in promoting RecA protease activity in vitro (27, 44). dATP and ATP were by far the most effective; the efficiencies of dUTP, UTP, dCTP, and CTP are about 10, 5, 0.1, and 0.1%, respectively, of that of dATP; GTP and dGTP only promote trace amounts of RecA protease activity; and TTP promotes no RecA protease activity. In fact, dUTP, UTP, CTP, dCTP, GTP, dGTP, or TTP can inhibit ATP-promoted RecA protease activity in vitro (27, 44; E. M. Phizicky, Ph.D. thesis, Cornell University, 1983) and thus could serve as negative effectors in vivo. It should be emphasized that the negative effectors are not inhibitors of RecA protease activity per se, but are competitive inhibitors that exclude the positive effector dATP or ATP from their binding site (13, 46, 47). Under normal growth conditions, the binding of negative NTP effectors may be responsible for the low wild-type RecA protease activity. However, a mutant RecA that preferentially binds positive effectors such as dATP or ATP would be expected to express protease activity constitutively. This kind of protease-constitutive *recA* mutant is most likely to have an altered NTP-binding site in its RecA proteins.

Previously we isolated many novel *recA* protease-constitutive (Prt^c) mutants, i.e., mutants in which the RecA protein is always in the protease state without the need for DNA-damaging agents to activate it (40, 41). Some of the *recA* (Prt^c) mutants are recombinase positive and thus are termed *recA* (Prt^c Rec^+) mutants. Other *recA* (Prt^c) mutants are recombinase defective and thus are termed *recA* (Prt^c Rec^-) mutants. We expected that the mutations of these Prt^c mutants would allow us to define ssDNA- and NTP-binding domains of RecA. In this paper, we describe the sequencing of these mutant *recA* genes. We found that their mutations clustered at three regions on the linear RecA polypeptide; thus these three regions of RecA may be involved in effector binding. By sequencing Prt^c Rec^- mutants we also showed that protease and recombinase functions of RecA overlap and that there is no complete separation of domains for these two functions. Finally, we describe the isolation of a promoter-down mutation that suppressed the RecA protease activity of one of our strong Prt^c mutants.

MATERIALS AND METHODS

Enzymes and chemicals. Restriction endonucleases were from Bethesda Research Laboratories, Inc., and Boehringer Mannheim Biochemicals; calf intestine alkaline phosphatase was from Boehringer Mannheim; T4 DNA ligase, the Klenow fragment of *E. coli* DNA polymerase I, M13 sequencing primers, and dideoxy and deoxynucleotide triphosphates were purchased from Bethesda Research Laboratories; [α-^{32}P]dATP (800 Ci/mmol) was from Amersham Corp.

Bacterial strains, phages, and media. Most of the bacterial strains, bacteriophages and media used in this work were described previously (40, 41). The *recA* mutants were all constructed by using λ *recA* cI *ind* mutant phages to lysogenize strain EST1515 [*ΔrecA306 sulA11 dinD1*::Mu d(Ap *lac*) *supE44*]. The wild-type strain was EST1450 [EST1515(λ *recA*$^+$ cI *ind*)]. Other strains used were EST1625 [EST1515 (ϕ21)]; IT1865 [as EST1515, but *lexA71* (Def)]; EST1122 [as EST1515, but Tet^s]; EST2512 [EST1515 (λ *recA1213-recA1611* cI *ind*)]; EST2582 [EST1515(λ *recA1213-recA1612* cI *ind*)]; EST1816 [EST1515(λ *recA1213* cI *ind*)]; EST1895 [IT1865(λ *recA1213* cI *ind*)]; EST2586 [IT1865(λ *recA1213-recA1611* cI *ind*)].

Isolation and characterization of *recA* mutants, measurement of β-galactosidase synthesis, measurement of recombination frequencies, and UV irradiation. The procedures used were as described previously (40, 41).

DNA manipulations. Standard techniques (18) were used. Restriction endonuclease digests, alkaline phosphatase treatments, and ligations were carried out as recommended by the manufacturers.

DNA sequence determination. Large amounts of λ cI *ind* phages carrying either wild-type or mutant *recA* genes were prepared according to standard methods (18) by using *E. coli* SA820, which is Δatt^{λ}, as a host strain. λ DNA that contained the *recA* gene was prepared (18) and digested with *Eco*RI. A 1.8-kilobase (kb) and a 1.3-kb *Eco*RI fragment, which contained three-fourths (N terminal) and one-fourth (C terminal) of the *recA* gene, respectively, were isolated and further digested with appropriate restriction endonucleases. The DNA fragments that contained parts of the *recA* gene were sequenced by inserting the purified fragments into M13 mp10 and mp11. Sequencing was by the dideoxy chain termination method of Sanger et al. (37) and carried out as recommended by the manufacturer (Bethesda Research Laboratories). The DNA sequence of our parental *recA*$^+$ gene was identical with the sequence previously reported for the wild-type *recA* (8, 10, 36).

Resolution of the *recA1201* double mutant into its single-mutant components *recA1602* and *recA1601*. The construction of plasmids containing either *recA1602* or *recA1601* is shown in Fig. 1. The general strategy was to construct two intermediate plasmids, one (pWB134) containing the C-terminal one-fourth of the *recA*$^+$ gene and the other (pWB176) containing the N-terminal three-fourths. In both cases the plasmids were constructed by ligating parts of pBR322 to purified fragments of pDR1453, which contains 8.6 kb of the *E. coli* chromosome including the *recA*$^+$ gene (35). *E. coli* W3110 was transformed with each of the two constructed plasmids, and transformants were selected by their Tet^r phenotype. Plasmid DNA was screened by restriction enzyme analysis to confirm the presence of a 1.3-kb *Eco*RI-*Pst*I insert in pWB134 and a 1.8-kb *Eco*RI-*Pvu*I insert in pWB176.

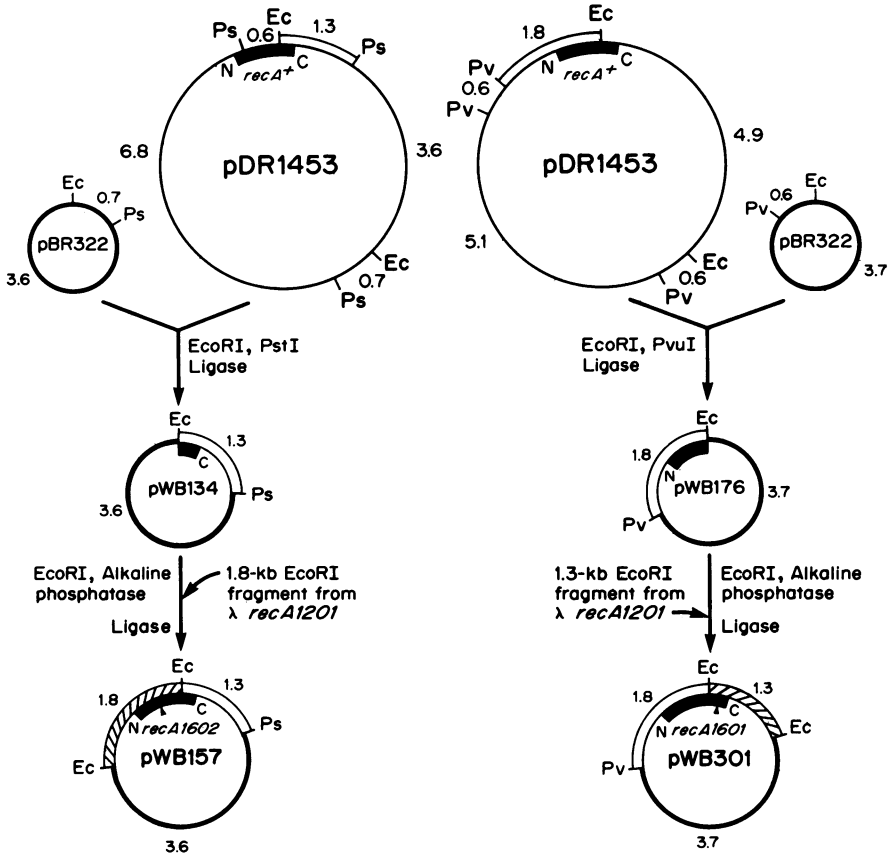

FIG. 1. Construction of plasmids pWB157 and pWB301, which contain, respectively, the N-terminal and C-terminal mutations of *recA1201*. Symbols: ▭, DNA fragment from pDR1453; ▨, DNA fragment from λ *recA1201*; ▬, coding region of *recA* gene; ▬, DNA fragment from pBR322; N and C, termini of the *recA* gene; ▲, a mutated site. The numbers between restriction sites indicate lengths of restriction fragments in kilobases. Abbreviations: Ec, *Eco*RI; Ps, *Pst*I; Pv, *Pvu*I.

The two intermediate plasmids were cut with *Eco*RI. The 1.8-kb N-terminal *Eco*RI fragment of λ *recA1201*, which contains the *recA1602* mutation, was ligated to pWB134 to form the reconstituted *recA1602* mutant in pWB157. The 1.3-kb C-terminal *Eco*RI fragment of λ *recA1201*, which contains the *recA1601* mutation, was ligated to pWB176 to form the reconstituted *recA1601* mutant in pWB301.

EST1122 was transformed with the two single mutant *recA* plasmids and spread on 5-bromo-4-chloro-3-indolyl-D-galactopyranoside–M9–Casamino Acids (CAA) plates containing 20 μg of tetracycline and 50 μg of ampicillin per ml and incubated at 35°C. Two types of colonies appeared on the selection plates: blue (~70%) and gray-blue (~30%). The blue colonies were expected to contain the reconstructed mutant *recA* because pWB134 and pWB176 transformants, which contain incomplete *recA* genes, make gray-blue colonies. Analysis of the plasmid DNA from a blue colony confirmed that pWB157 contained a 1.8-kb *Eco*RI insert and that pWB301 contained a 1.3-kb *Eco*RI insert. DNA sequence analysis showed that the *recA* gene of pWB157 has a single mutation, which changes Gly-157 to Asp, and the *recA* gene of pWB301 has a single mutation, which changes Gly-301 to Ser.

Finally the mutant *recA* genes in pWB157 and pWB301 were inserted into phage λ by ordinary genetic recombination (6). Transformed cells were infected with UV-irradiated phage at a multiplicity of infection of 1, unadsorbed phage were removed by centrifugation, and the cells were diluted with LB broth and incubated at 37°C for 90 min. The progeny phage were plated on 5-bromo-4-chloro-3-indolyl-D-galactopyranoside–M9–CAA plates seeded with EST1515. In the case of pWB157, infection was with λ *recA1213* cI *ind*. The recombinant phage produced pale blue plaques in contrast to the background of dark blue plaques produced by the parental phage. Phage from a pale blue plaque was shown by sequence analysis to contain the *recA1602* gene. In the case of pWB301, infection was with λ *recA*$^+$ cI *ind*. The recombinant phage produced blue plaques in contrast to the background of gray-blue plaques produced by the parental phage. Phage from a blue plaque was shown by sequence analysis to contain the *recA1601* gene. The choice of the λ strain used in the crosses was determined by the need to get strong color contrast between the recombinant phage and the parental phage. The *recA1602* mutant has weak Prt^c activity and therefore required a strong Prt^c phage like λ *recA1213* for contrast.

Isolation of suppressor mutants. λ *recA1213* cI *ind* phage were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described previously (40). The mutagenized phage were plated on 5-bromo-4-chloro-3-indolyl-D-galactopyranoside–M9–CAA plates seeded with the φ21 lysogen Δ*recA dinD::lac* strain EST1625 and incubated at 35°C. Pale blue turbid and gray turbid plaques were found among the dark blue clear plaques that were produced by the parental λ *recA1213* cI *ind* phage. Phage from pale blue turbid, rather than gray turbid, plaques were picked and

TABLE 1. Nucleotide changes and resulting amino acid substitutions of *recA* (Prt^c) mutants

Allele	Phenotype[a]	Base pair no.[b]	Condon change	Residue no.	Residue change
recA1202	Prt^c Rec$^+$	602	CAG → AAG	184	Gln → Lys
recA1212	Prt^c Rec$^+$	588	GCG → GTG	179	Ala → Val
recA1213	Prt^c Rec$^+$	588	GCG → GTG	179	Ala → Val
recA1219	Prt^c Rec$^+$	524	GAA → AAA	158	Glu → Lys
		1,133	G → A[c]		
recA1211	Prt^c Rec$^+$	164	GAA → AAA	38	Glu → Lys
recA1222	Prt^c Rec$^+$	126	TCC → TTC	25	Ser → Phe
recA1235	Prt^c Rec$^+$	168	ACC → ATC	39	Thr → Ile
recA1602	Prt^c Rec$^\pm$	522	GGC → GAC	157	Gly → Asp
recA1601	Prt^c Rec$^-$	953	GGT → AGT	301	Gly → Ser
recA1203	Prt^c Rec$^-$	557	CGT → TGT	169	Arg → Cys
		436	ATC → ATT	128	Ile → Ile
recA1206	Prt^c Rec$^-$	954	GGT → GAT	301	Gly → Asp
		583	AAG → AAA	177	Lys → Lys
recA1207	Prt^c Rec$^-$	954	GGT → GAT	301	Gly → Asp
recA1201	Prt^c Rec$^-$	522	GGC → GAC	157	Gly → Asp
		953	GGT → AGT	301	Gly → Ser

[a] For a description of the phenotypes, see previous paper (40).
[b] Numbering is from the transcriptional start of the *recA* gene (8).
[c] Nucleotide change occurred outside coding region of *recA*.

purified because most phage from gray turbid plaques were expected to have severe defects in their RecA proteins (40, 41), whereas phage from pale blue turbid plaques were expected to encode RecA proteins that could fold more or less properly because they retained at least some protease activity (although not enough to induce ϕ21). The purified phage from pale blue turbid plaques were further characterized after lysogenization of strain EST1515.

RESULTS

Clustering of the amino acid changes of *recA* (Prt^c) mutants in three regions of the linear RecA polypeptide. In earlier work (40, 41), we described the isolation of many *recA* (Prt^c) mutants and characterized the protease activities of these mutants by five indices: (i) specific activity of β-galactosidase expressed constitutively from a *dinD::lac* fusion gene; (ii) efficiency of spontaneous cleavage of the repressor of λ *imm*^{434}; (iii) sensitivity to mitomycin C, crystal violet, antibiotics, and broth; (iv) frequency of spontaneous mutagenesis; and (v) ability of the constitutive protease activity to be inhibited by C+G.

In sequencing the DNA of these *recA* (Prt^c) mutants, we usually selected for study the *recA* (Prt^c) mutants with the most distinctive phenotypes. The nucleotide sequence of the whole *recA* gene including the regulatory region was determined. The nucleotide and amino acid substitutions found in 13 mutants are shown in Table 1. All of the *recA* (Prt^c) mutants contained a single amino acid change, except *recA1201*, which contained two amino acid changes. All of the mutations were transitions except for the transversion that produced the *recA1202* mutant; this is consistent with evidence that nitrosoguanidine treatment primarily causes transitions (23).

The positions of amino acid changes of these *recA* (Prt^c) mutants and their clustering in three regions are shown on a linear RecA polypeptide (Fig. 2). Region 1 includes amino acid residues 25 through 39, region 2 includes amino acid residues 157 through 184, and region 3 includes amino acid residues 298 through 301. Since *RecA* proteins of Prt^c mutants may have been altered in their effector-binding sites (see above and Discussion), these polypeptide regions may be involved in effector binding.

Evidence for involvement of polypeptide region 1 in ssDNA binding and polypeptide region 2 in NTP binding. C+G is known to inhibit in vivo protease activity of the *recA441* mutant, a temperature-dependent protease-constitutive mutant (11, 39, 52). It was proposed that derivatives of C+G formed in vivo may serve as negative effectors that compete for the same NTP-binding site as the positive effectors ATP and dATP (27, 39). It is possible that *recA* mutants that are altered in the NTP-binding site such that their RecA proteins have lower affinity for derivatives of C+G would be inhibited less by C+G. We compared the effect of C+G on the constitutive protease activity of our strong Prt^c mutants. These strong protease-constitutive mutants would be expected to have the most striking changes in the effector-binding site. The constitutive protease activities of *recA1202*, *recA1212*, *recA1213*, and *recA1219* were inhibited either slightly or not at all by C+G; these mutations occurred in region 2 (Table 2). We propose that the NTP-binding sites of these mutants have been altered such that their affinity for negative effectors, derivatives of C+G, is much reduced, and that region 2 is involved in NTP binding. In contrast to region 2 mutants, protease activities of the region 1 mutants *recA1211*, *recA1222*, and *recA441* were much more sensitive to C+G inhibition (Table 2). Constitutive protease activities of both the *recA1222* and *recA441* mutants were inhibited to the basal level of the *recA*$^+$ strain.

RecA441 protein has been shown to have two amino acid changes (Fig. 2) (12), one at codon 38 (Glu → Lys), the other at codon 298 (Ile → Val). The amino acid change at codon 38 was shown to be responsible for the constitutive protease activity of the *recA441* strain, whereas the change at codon 298 suppressed the codon 38 mutation in a temperature-dependent way (43). RecA441 protein shows higher affinity for single-stranded oligonucleotides than does the wild-type RecA protein (20, 27) and was proposed to have an altered ssDNA-binding site (20). It is conceivable that the amino acid change Glu → Lys at codon 38 in both *recA441* and *recA1211* strains increases the affinity of RecA for oligonucleotides because this change increases the positive charge of the RecA protein. The *recA1222* mutation changes serine at codon 25 to phenylalanine, a change that may increase the affinity of RecA for ssDNA because aromatic amino acids can interact with oligonucleotides more strongly than do nonaromatic amino acids. We propose that RecA proteins of these mutants have an altered ssDNA-binding site and therefore that region 1 of the RecA protein is involved in ssDNA binding. This conclusion has been reached from two additional observations (10): (i) the N-terminal region of RecA protein is homologous to the N-terminal regions of both the *E. coli* ssDNA-binding protein and the phage T4 gene 32 protein, both of which have been shown to bind to ssDNA; (ii) RecA protein becomes defective in its ability to bind ssDNA when 33 amino acids are removed from its N terminus.

Evidence that the protease and recombinase domains of the RecA protein overlap. Transductional mapping data (24) and the behavior of truncated RecA proteins (53) suggested that the protease and recombinase activities of the RecA protein lie in separate domains, but whether domains are overlapping or separated has not been proved definitely. We sought to settle this question by sequencing the DNAs of our *recA* (Prt^c Rec$^-$) and *recA* (Prt^c Rec$^\pm$) mutants. The constitutive protease activity and recombinase activity of these Prt^c Rec$^-$ and Prt^c Rec$^\pm$ mutants are shown in Table 3. All Prt^c Rec$^-$

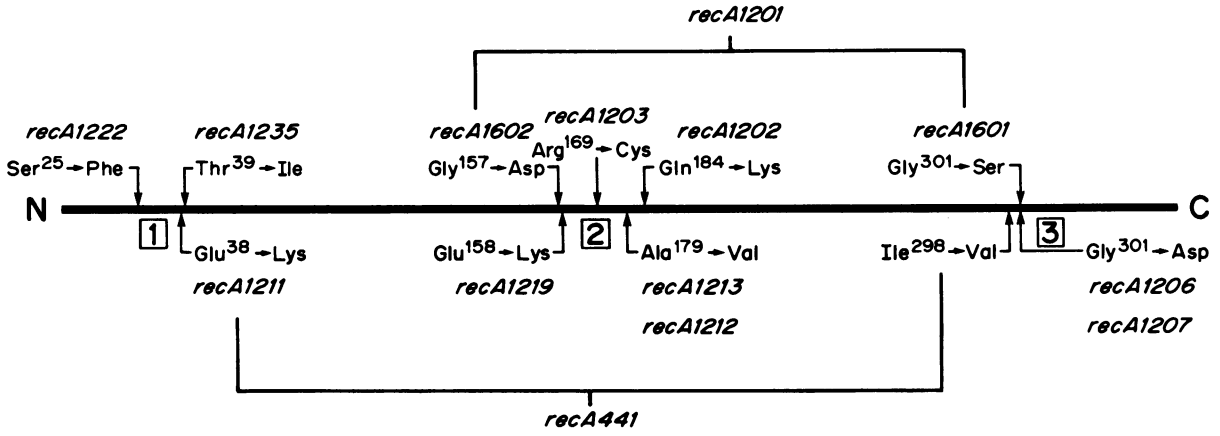

FIG. 2. Mutational map of the RecA protein.

mutants have higher constitutive protease activity and much less recombinase activity than the wild-type strain, whereas *recA1602* has slightly lower recombinase activity than the wild-type strain. DNA sequencing showed that all of these mutants have single amino acid changes, except *recA1201*, which contains two amino acid changes (Table 1). These results indicated that single amino acid changes can affect both protease and recombinase functions of RecA, and therefore functional domains for protease and recombinase activities of RecA must overlap. The amino acid changes of these Prt^c Rec$^-$ and Prt^c Rec$^\pm$ mutants occur in regions 2 and 3 (Fig. 2), indicating that the protease and recombinase domains of RecA overlap at least in these two regions.

Evidence that polypeptide region 2 interacts with region 3 in the tertiary structure of RecA. By using affinity labels 8-azidoadenosine 5′-triphosphate and 5′-*p*-fluorosulfonylbenzoyladenosine, Knight and McEntee (13, 14) concluded that amino acid residue 264, which is close to region 3, is involved in NTP binding. We found that polypeptide region 2, which spans residues 157 to 184, may be involved in NTP binding. How can these two different findings be reconciled? We compared the phenotypic differences between the double mutant *recA1201* and its single-mutant components *recA1602* and *recA1601*. The *recA1201* mutant contains two amino acid changes, one at region 2 and the other at region 3 (Fig. 2). We separated the two mutations by cloning each one into the derivatives of pBR322, from where each mutation was crossed into λ (see Materials and Methods). In this way we were able to compare the phenotypes of the double mutant and its single-mutant components on single-copy λ prophages.

All three mutants had constitutive protease activity. The constitutive protease activities of *recA1201* and *recA1601* were similar, but that of *recA1602* was slightly lower (Table 3). Both single mutants were partially UV sensitive, *recA1601* being much more UV sensitive than *recA1602*, whereas the double mutant *recA1201* was very UV sensitive (data not shown). The UV sensitivity of the mutants corresponded to their recombinase activity (Table 3), a result consistent with the idea that recombinase activity is the major factor that accounts for UV sensitivity (28, 41).

Previously (40), we showed that *recA1201* is an anomalous mutant in that the inhibitory effect of C+G cannot be reversed by mitomycin C. We were curious about whether this anomalous response to effectors also occurs in the single mutants. The effect of C+G on the constitutive protease activity of double and single mutants and its reversibility by mitomycin C are shown in Table 4. In the presence of C+G,

TABLE 2. Effect of C+G on the constitutive RecA protease activities of strong *recA* (Prt^c) mutants

Strain	Allele	Polypeptide region at which amino acid change occurs	β-Galactosidase sp act[a,b] (U)	Inhibition by C+G[a,c]
EST1450	*recA*$^+$		10	UN[d]
EST1813	*recA1202*	2	184	−
EST1822	*recA1212*	2	165	−
EST1816	*recA1213*	2	158	−
EST1826	*recA1219*	2	150	+
EST1799	*recA1211*	1	174	+ +
EST1630	*recA1222*	1	170	+ + +
EST1130	*recA441*	1, 3	103	+ + +

[a] Data from Tessman and Peterson (40).

[b] β-Galactosidase specific activity measures the expression of the SOS gene *dinD*::*lac*, which in turn measures the protease activity of RecA protein semiquantitatively (40).

[c] Symbols: + + +, inhibited to the basal level of the *recA*$^+$ strain; + + and +, inhibited to 2.5 and 4 times the basal level, respectively; −, noninhibitable. It should be noted that there is always a nonspecific inhibition (about 40%) by C+G (40); therefore the Prt^c mutants which were termed noninhibitable actually showed a 40% decrease in the β-galactosidase specific activity in the presence of C+G.

[d] UN, Undefined because protease activity of the *recA*$^+$ strain is already at the basal level.

TABLE 3. Properties of *recA* (Prt^c Rec$^-$) and *recA* (Prt^c Rec$^\pm$) mutants

Strain	Allele	Phenotype	β-Galactosidase sp act[a] (U)	Relative recombination frequency[b]	Inhibition by C+G[c]
EST1450	*recA*$^+$	Prt$^+$ Rec$^+$	10	1.0	UN
EST1694	*recA1203*[d]	Prt^c Rec$^-$	69	2×10^{-4}	−
EST1454	*recA1206*[d]	Prt^c Rec$^-$	52	2×10^{-4}	−
EST1115	*recA1207*[d]	Prt^c Rec$^-$	58	1×10^{-4}	−
EST2450	*recA1601*	Prt^c Rec$^-$	65	5×10^{-3}	−
EST2581	*recA1602*	Prt^c Rec$^\pm$	38	3×10^{-1}	+ +
EST1446	*recA1201*	Prt^c Rec$^-$	60	1×10^{-4}	+ + +
EST2585	*recA1201*[e]	Prt^c Rec$^-$	64	NM	−

[a] Measured as described by Tessman and Peterson (40).

[b] Recombination frequencies were measured by liquid culture matings with an Hfr strain (40). NM, Not measured.

[c] As described in footnote *c* of Table 2, except that + + indicates inhibition to about two times the basal level of the *recA*$^+$ strain.

[d] Data from Tessman and Peterson (41).

[e] The *recA1201* allele was in a multicopy plasmid derived from pBR322 and transformed strain EST1122 to make strain EST2585.

TABLE 4. Effect of different effectors on the protease activities of the double mutant *recA1201* and its single-mutant components *recA1601* and *recA1602*

Strain	Allele	β-Galactosidase sp act[a] (U)			
		No effector	C+G	C+G and MT[b]	MT[b]
EST1446	*recA1201*	60	11	15	136
EST2450	*recA1601*	65	54	105	147
EST2581	*recA1602*	38	18	84	103
EST1450	*recA*$^+$	10	UN	89	101

[a] Measured as described by Tessman and Peterson (40). C, 300 μg of cytidine per ml; G, 350 μg of guanosine per ml; MT, 0.5 μg of mitomycin C per ml. UN, Undefined (see footnote *d* of Table 2).
[b] Values are the average plateau levels after induction by mitomycin C.

the constitutive protease activity of the double mutant *recA1201* was inhibited to the basal level of the *recA*$^+$ strain. In contrast, the constitutive protease activities of the single mutants *recA1601* and *recA1602* were inhibited less by C+G. The protease activities of both *recA1601* and *recA1602* could be induced by mitomycin C in the presence of C+G. In contrast, the protease activity of *recA1201* could not be induced by mitomycin C in the presence of C+G; i.e., the protease activity of *recA1201* remained at the basal level when mitomycin C was added in the presence of C+G.

The response of the single mutants, *recA1601* and *rec1602*, to mitomycin C in the presence of C+G is normal compared with that of other *recA* (Prt^c) mutants (40), whereas *recA1201* is the only mutant that has the anomalous response. The fact that the double mutant *recA1201* is different from each of its single-mutant components and other *recA* (Prt^c) mutants in responding to effectors indicates that the RecA protein of the double mutant *recA1201* has an unusual effector-binding site that is different from those of single mutants *recA1601* and *recA1602*. We propose that the two amino acid changes (at residues 157 and 301) found in the double mutant *recA1201* somehow interact with each other in the tertiary structure of RecA, creating an unusual effector-binding site. Since one amino acid change occurs in polypeptide region 2 and the other occurs in region 3 of RecA, we infer that regions 2 and 3 may interact in the tertiary structure of RecA and together may comprise part of an effector-binding domain.

Promoter mutation that suppressed constitutive protease activity of the strong *recA* (Prt^c) mutant *recA1213*. We were interested in isolating suppressor mutations of our strong *recA* (Prt^c) mutants, since such mutations would provide more information about how polypeptide regions may interact. λ *recA* cI *ind* containing the strong Prt^c mutation *recA1213* was mutagenized with nitrosoguanidine and then plated on 5-bromo-4-chloro-3-indolyl-D-galactopyranoside–M9–CAA plates seeded with the φ21 lysogen Δ*recA dinD::lac* strain EST1625. Pale blue turbid plaques were found among dark blue clear plaques (see Materials and Methods). Phage from these pale blue turbid plaques presumably encode RecA proteins whose constitutive protease activity is much reduced relative to that of the original RecA1213 protein. *recA* DNA from four of these suppressor mutants were sequenced. The nucleotide and amino acid sequences were compared with those of their parental *recA1213* strain (Table 5). Three of the four suppressor mutants had the same suppressor mutation at 32 base pairs upstream from the transcriptional initiation site. This mutation is called *recA1611*. Another suppressor mutation, called *recA1612*, occurred at codon 16 and changed glutamine to an amber nonsense codon.

TABLE 5. Nucleotide changes and resulting amino acid substitutions of suppressor mutations of the strong Prt^c mutant *recA1213*

Allele[a]	Base pair no.[b]	Nucleotide change	Residue no.	Residue change
recA1213	588	C → T	179	Ala → Val
recA1213-recA1611	588	C → T	179	Ala → Val
	−32	G → A		
recA1213-recA1613	588	C → T	179	Ala → Val
	−32	G → A		
	53	G → A	1	Ala → Thr
	449	G → A	133	Ala → Thr
recA1213-recA1612	588	C → T	179	Ala → Val
	98	C → T	16	Gln → Amb

[a] Four independent suppressor mutants were sequenced, among which two isolates had the same nucleotide changes and were named *recA1213-recA1611*.
[b] Numbering is from the transcriptional start of the *recA* gene (8).

The effect of the *recA1611* and *recA1612* mutations on the expression of RecA function was studied in the strains EST2512, which is *recA1213-recA1611*, and in EST2582, which is *recA1213-recA1612*. Both strains EST2512 and EST2582 contain a *supE44* mutation, which suppresses amber mutations by translating the nonsense codon as glutamine. The constitutive protease activity of the strain EST2582 was suppressed about sixfold relative to that of the parental strain EST1816, which contained only the *recA1213* mutation (Table 6). Since the EST2582 strain contained an amber mutation in its *recA* gene and only about 20% readthrough of RecA is possible in this strain (2), the suppression of RecA constitutive protease activity must be due to the lower amount of RecA protein synthesized in strain EST2582. The constitutive protease activity of the EST2512 strain was also suppressed about sixfold relative to that of the parental strain EST1816 (Table 6). But the *recA1611* mutation that is found in strain EST2512 does not affect the structure of the RecA protein; therefore the only explanation for the suppression is that EST2512, like EST2582, produced less RecA protein than did the parental strain EST1816. The reduction of RecA protein synthesis in EST2512 must have been due to the *recA1611* mutation, which occurs at −32 in the *recA* promoter region (48, 49). We propose that the *recA1611* mutation is a promoter-down mutation and thus is similar to the *recA453* mutation (4, 25).

The conclusion that the *recA1611* mutation is a promoter-down mutation was also suggested by the survival of UV-irradiated EST2512. The UV survival curves of strains EST2512 and EST2582 were compared with those of EST1816 and EST1450 (Fig. 3). Both EST2512 and EST2582 were much more UV sensitive than EST1816 and EST1450, indicating that less RecA protein was produced in EST2512 and EST2582 than in EST1816 and EST1450. Since amplification of the RecA protein is essential for cell survival after UV irradiation (28), this result also showed that the RecA

TABLE 6. Constitutive RecA protease activities of suppressor mutants and their parental strain *recA1213*

Strain	Allele	β-Galactosidase sp act[a] (U)
EST1816	*recA1213*	166
EST2512	*recA1213-recA1611*	28
EST2582	*recA1213-recA1612*	26
EST1450	*recA*$^+$	12

[a] Measured as described by Tessman and Peterson (40).

protein could not be properly amplified in EST2512 and EST2582 after UV irradiation, indicating that their mutations, *recA1611* and *recA1612*, respectively, prevented the amplification.

DISCUSSION

By characterizing our *recA* (Prt^c) mutants and finding by sequencing their DNA that they are clustered, we identified three regions of the RecA polypeptide that could be associated with specific functions. Region 1 (amino acids 25 through 39) appears to be involved in ssDNA binding; region 2 (amino acids 157 through 184) appears to be involved in NTP binding; region 3 (amino acids 298 through 301) appears to be involved in both ssDNA and NTP binding. We used function-enhanced rather than function-deficient mutants to identify RecA functional sites, because we expected these function-enhanced *recA* (Prt^c) mutants to produce proteins specifically altered at functional sites.

An important aid in the identification of the NTP-binding regions was the quantifiable effect that the addition of C+G to the medium had on the protease activity of various *recA* (Prt^c) mutants. C+G in the growth medium is known to inhibit the protease activity of the RecA441 protein (11, 39, 40, 52), but the precise reason is not known. However, there is evidence that the addition of free bases in the growth medium can affect the ratio of NTPs in the cell (33), so it is possible that C+G increases the concentration of negative NTP effectors relative to that of positive NTP effectors (39). Evidence from our study of the *recA1201* allele, a Prt^c Rec$^-$ mutant, indicated that derivatives of C+G can directly interact with the RecA proteins. When a single copy of the *recA1201* gene was present in the cell, the constitutive RecA protease activity of the cell could be inhibited by C+G (Tables 3 and 4), but when multicopies of the *recA1201* gene were present in an isogenic strain, the constitutive RecA protease activity of the cell was no longer inhibited by C+G (Table 3). Apparently, an increased amount of RecA protein in the cell can titrate away the effect of C+G, indicating a direct interaction of C+G derivatives with RecA protein.

The constitutive protease activities of region 2 mutants were only partially, if at all, inhibited by adding C+G to the growth medium, suggesting that the RecA proteins of these mutants have an increased preference for positive relative to negative NTP effectors, or that derivatives of C+G may behave as positive effectors for these mutants. In either case, the mutational changes of these mutants would occur in the NTP-binding domain.

The double mutant *recA1201* differed from each of its single-mutant components, *recA1601* and *recA1602*, and other *recA* (Prt^c) mutants in its response to effectors (Table 4) (40), showing that the RecA1201 protein obtains its unusual effector-binding site by combining the mutational effects of its two components. The protease activity of the *recA1201* strain was induced to the same extent as the *recA*$^+$ strain when induction was by mitomycin C in the absence of C+G (Table 4), indicating that the ssDNA-binding site of the RecA1201 protein is normal. But when *recA1201* cells were grown in the presence of C+G the protease activity could not be induced by mitomycin C treatment, in sharp contrast to the behavior of *recA*$^+$ cells, which showed high protease activity under those conditions (Table 4). We conclude, therefore, that the region 2 and region 3 components combine to form a RecA1201 protein that has an altered NTP-binding site with an unusual preference for negative NTP effectors, derivatives of C+G. The binding of negative NTP

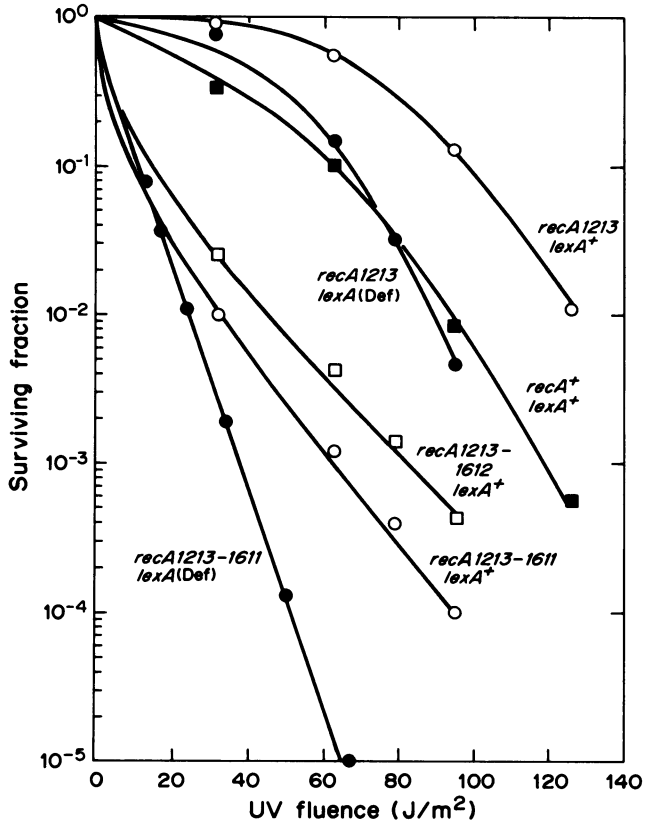

FIG. 3. UV survival curves of the *recA1213*, *recA1213-recA1611*, and *recA1213-recA1612* alleles in a *lexA*$^+$ background and in an isogenic *lexA71*(Def) background. Strains: EST1450 *recA*$^+$ *lexA*$^+$; EST1816 *recA1213 lexA*$^+$; EST1895 *recA1213 lexA71*(Def); EST2512 *recA1213-recA1611 lexA*$^+$; EST2586 *recA1213-recA1611 lexA71*(Def); EST2582 *recA1213-recA1612 lexA*$^+$.

effectors causes RecA to have a low affinity for ssDNA (19, 21, 22; Phizicky, Ph.D. thesis), which may explain why RecA1201 protease could not be activated even after mitomycin C treatment.

The conclusion that region 3, as well as region 2, is involved in NTP binding was also suggested by the phenotypes of the region 3 mutants *recA1206*, *recA1207*, and *recA1601*. As with region 2 mutants, the constitutive protease activity of these mutants could not be inhibited by C+G (Table 3), indicating that the NTP-binding sites of these mutants might have been altered to a state with reduced affinities for C+G-derived negative NTP effectors. The involvement of region 3 in NTP binding is also supported by the finding that Tyr-264 of the RecA protein may be the ATP attachment site (13, 14).

We propose that regions 1 and 3 together may comprise part of the ssDNA-binding domain of RecA. The constitutive protease activities of region 1 mutants were inhibitable by C+G. One of these mutants, *recA441*, has been proposed to have an altered ssDNA-binding site (20). Although *recA441* has two mutations (12), the one which is responsible for its constitutive protease activity alters codon 38 (43), which alone would therefore presumably alter ssDNA binding. But the second mutation in codon 298 in region 3 is a temperature-dependent suppressor of the codon 38 mutation in region 1 (43). This implicates region 3, like region 1, in ssDNA binding.

Our inference that ssDNA-binding and NTP-binding domains of RecA overlap in region 3 may explain the complex allosteric effects produced by effector binding. The binding of NTP affects RecA affinity for ssDNA (19, 21, 22; Phizicky, Ph.D. thesis), and conversely the binding of ssDNA affects RecA affinity for NTP (5, 19, 47). That the allosteric interaction between ssDNA binding and NTP binding may be coupled through the C-terminal region of RecA was also proposed by Blanar et al. (1). The involvement of region 3 in both ssDNA and NTP binding is supported by the observation that a truncated RecA protein, which consists of 60% of the N-terminal region of RecA, can no longer bind ssDNA and NTP (34).

How could altered effector-binding sites cause RecA protease to be constitutively active in the cell? In vitro, RecA affinity for ssDNA is much higher in the presence of positive nucleotide effectors than in the presence of negative nucleotide effectors (19, 21, 22; Phizicky, Ph.D. thesis). Under normal growth conditions RecA protease would not be activated, because the affinity of RecA for ssDNA, influenced by the average effects of the nucleotide effectors present in the cell, would be expected to be too low to compete with ssDNA-binding protein for the limited amount of ssDNA available. RecA protein could become constitutively activated by increasing its affinity for ssDNA. This could be achieved directly by an alteration of the ssDNA-binding site. It also could be achieved indirectly by an alteration of the NTP-binding site that would make it preferentially bind positive NTP effectors; this would then create a higher affinity for ssDNA at the ssDNA-binding site. In either way the protease activity could be activated without DNA-damaging treatment.

We cannot rule out the possibility that for some of our mutants the RecA site that is directly involved in proteolysis of LexA repressor may be altered in such a way that the protein would be constitutively active without the need for ssDNA and NTP for activation. If such mutants do exist, their protease activity would not be affected by adding effectors such as C+G, adenine, or mitomycin C to the growth medium. The fact that the protease activity of some of our Prt^c mutants could be inhibited by C+G (Table 2; 40) and also that the inhibitory effect of C+G could be partially reversed by adding adenine or mitomycin C to the growth medium (40; unpublished data) indicated that some of these mutants were actually altered in the effector-binding sites rather than sites directly involved in repressor cleavage. We are currently analyzing these mutants biochemically for direct proof that our Prt^c mutants are altered in the effector-binding sites. Preliminary in vitro studies of the *recA1201*, *recA1202*, *recA1211*, *recA1213*, *recA1219*, and *recA1222* mutants indicate that their RecA proteins still depend on the ssDNA and NTP effectors for their protease activity and that the RecA1202 protein binds ATP more strongly than does wild-type RecA protein (W.-B. Wang, E. S. Tessman, M. Sassanfar, and J. W. Roberts, unpublished data).

It has been suggested that RecA protein has separate domains for its protease and recombinase functions (24, 53). We found this was not the case from the DNA sequence analysis of our Prt^c Rec$^-$ and Prt^c Rec$^\pm$ mutants. Each of five mutants had single amino acid changes, indicating that the functional domains for both protease and recombinase activities of RecA overlap. Weigle repair and UV-induced mutagenesis were normal, but the frequency of spontaneous mutagenesis was reduced in our Prt^c Rec$^-$ mutants (E. S. Tessman, I. Tessman, P. K. Peterson, and J. D. Forestal, unpublished data). Protease activation and an early stage of recombinase function may both require the same ternary complex of RecA protein, ssDNA, and ATP (5, 30, 38, 41). Since our Prt^c Rec$^-$ mutants have protease activity, they may be able to initiate recombination, but their recombinase defects caused by changes at codons 157, 169, and 301 should affect later stages of recombination, including hydrolysis of ATP and branch migration.

In the course of isolating suppressor mutants of *recA1213*, a strong *recA* (Prt^c) mutant, we found that the mutation *recA1611* at position −32 can reduce the amount of RecA protein synthesized in the cell. Since the *recA1611* mutation occurs within the promoter region and in the proximity of the LexA-binding site of *recA* (48, 49), two mechanisms can possibly explain the effect of the *recA1611* mutation on RecA protein synthesis. (i) The *recA1611* mutation might affect the operator activity of the gene so that LexA repressor would bind to this mutant operator more tightly than to the wild-type operator. (ii) The *recA1611* mutation might affect the promoter activity of the gene so that RNA polymerase would have a lower affinity for this mutant promoter than for the wild-type promoter. We sought to distinguish between these two mechanisms by comparing the UV survival curves of the mutant *recA1213-recA1611 lexA*$^+$, *recA1213-recA1611 lexA*(Def), *recA1213 lexA*$^+$, and *recA1213 lexA*(Def) strains. We found that both *recA1213-recA1611 lexA*$^+$ and *recA1213-recA1611 lexA*(Def) strains were much more UV sensitive than the isogenic *recA1213 lexA*$^+$ and *recA1213 lexA*(Def) strains (Fig. 3). If the *recA1611* mutation affected only the binding of LexA repressor to the *recA* operator, then the *recA1213-recA1611 lexA*(Def) strain would be as UV resistant as the *recA1213 lexA*(Def) strain, since there is no LexA repressor present in the cell. The finding that the *recA1213-recA1611 lexA*(Def) strain is very UV sensitive indicates that the *recA1611* mutation affects promoter rather than operator activity of the *recA* gene and thus is a promoter-down mutation. The method used here for isolating suppressor mutants could prove to be useful in isolating promoter mutations of the *recA* gene, since three out of four suppressor mutants turned out to have a promoter-down mutation.

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