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Genetic Characteristics of New *recA* Mutants of *Escherichia coli* K-12

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To search for functionally thermosensitive (FT) *recA* mutations, as well as mutations with differently affect RecA protein functions, seven new *recA* mutations in three different regions of the RecA protein structure proposed by Story et al. [R. M. Story, I. T. Weber, and T. A. Steitz, Nature (London) 355:318–325, 1992] were constructed. Additionally, the *recA2283* allele responsible for the FT phenotype of the *recA200* mutant was sequenced. Five single mutations (*recA2277*, *recA2278*, *recA2283*, *recA2283E*, and *recA2284*) and one double mutation (*recA2278-5*) generated, respectively, the amino acid substitutions L-277→N, G-278→P, L-283→P, L-283→E, I-284→D, and G-278→T plus V-275→F in the α-helix H–β-strand 9 region of the C-terminal domain of the RecA protein structure. According to recombination, repair, and SOS-inducible characteristics, these six mutations fall into four phenotypic classes: (i) an FT class, with either inhibition of all three analyzed functions at 42°C (*recA2283*), preferable inhibition at 42°C of recombination and the SOS response (*recA2278*), or inhibition at 42°C of only recombination (*recA2278-5*); (ii) a moderately deficient class (*recA2277*); (iii) a nondeficient class (*recA2283E*); and (iv) a mutation with a null phenotype (*recA2284*). The *recA2223* mutation generates an L-223→M substitution in β-strand 6 in a central domain of the RecA structure. This FT mutation shows preferable inhibition of the SOS response at 42°C. The *recA2183* mutation produces a K-183→M substitution in α-helix F of the same domain. The Lys-183 position in the *Escherichia coli* RecA protein was found among positions which are important for interfilament interaction (R. M. Story, I. T. Weber, and T. A. Steitz, Nature (London) 355:318–325, 1992).

In vivo, the RecA protein of *Escherichia coli* plays a critical role in such cellular functions as homologous recombination, repair of damaged DNA, and the SOS response (see references 10, 12, and 21 for reviews). To mediate necessary biochemical reactions, the RecA protein must be in an active form that combines three ingredients (RecA, ATP, and single-stranded DNA) in a helical RecA nucleoprotein filament (17).

The current view of the physical structure of the filament is based on X-ray analysis of RecA crystals (23). According to that analysis, the RecA monomer is composed of three domains, one major domain and two subdomains located at its amino and carboxyl termini. Both the major domain and the N-terminal domain participate in the formation of RecA multimers, the structure of which has been suggested to resemble closely that of the RecA-DNA filament (23). The C-terminal domain protrudes from the multimer. While it does not participate in an intrafilament interaction, it does take part in an interfilament interaction. The latter has been recently discussed as a possible step in the recombination exchange reaction (13).

The C-terminal domain consists of three α helices (H, I, and J) that are situated orthogonally and two β strands (β9 and β10) located between the H and I helices (23). Epitope mapping of anti-RecA protein monoclonal immunoglobulins G, ARM191 and ARM193, which were suggested to affect, respectively, the site for interaction of RecA monomers within the RecA filament and the site for interaction between RecA and double-stranded DNA, showed that the antibodies cover the region of the C-terminal domain including at least β-strand 10, as well as helices I and J (7). The functional role of α-helix H and adjacent β-strand 9 remains unclear. We used site-directed mutagenesis to elucidate the possible functions of these two adjacent structures.

The other reason for our mutagenesis study was to search for functionally thermosensitive (FT) mutations. To date, only two FT mutations, *recA44* (8) and *recA200* (14), have been described. The former has been localized. We sequenced the *recA2283* allele, which was responsible for the RecA200 FT phenotype. The analysis of both *recA44* and *recA2283* mutations helped us to find a strategy for searching for other thermosensitive mutations in this and other regions. The rationale was to design amino acid substitutions which must be functionally related to the original residues.

Besides the C-terminal domain, two regions of the major domain, β-strand 6 and α-helix F, were involved in the construction of new mutations. The choice of the former region was dictated by an expectation, based on the properties of known *recA* mutations from this area, that it should be possible to reveal the site responsible for regulation of interaction between the RecA protein and the LexA repressor (see reference 19 for a review). The α-helix F region belongs to the type of polypeptide motifs predicted by Churchill and Travers (3) as that which might recognize structural features of DNA.

In this report, we describe the recombination, repair, and SOS response characteristics of eight *recA* mutations generating amino acid substitutions in three structural constituents of the RecA polypeptide including the αH-β9, β6, and αF regions.

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TABLE 1. Oligonucleotide primers used for PCR-based mutagenesis

Primer designation	Amino acid substitution(s) obtained	Mutant codon designed	Primer sequence
Type A[a]			
A1	K-183→M	**atg**	5′-gtaacctg**atg**cagtccaa
A2	L-223→M	**atg**	5′-tctgttcgt**atg**gacatcc
A3	L-277→N	**aac**	5′-tggttgac**aac**ggcgtaaaa
A4	G-278→P or G-278→T + V-275→F	**ccg** or **acg**	5′-ttgacctg**c**/**acg**gtaaaagag
A5	L-283→E	**gaa**	5′-aagagaag**gaa**atcgagaaa
A6	I-284→D	**gac**	5′-agaagctg**gac**gagaaagca
Other types			
B			5′-gattctacgattcgcactgtaggaaacagctatgaccatg
C			5′-gttttcccagtcacgacgtt
D			5′-gattctacgattcgcactgt

[a] A, B, C, and D are the primers used at different steps of the mutagenesis procedure developed previously (15).

MATERIALS AND METHODS

Strains and plasmids. All of the bacteria used in this study were derived from *E. coli* K-12. Strains KL227 (HfrP4x *metB*) and JC10289 [F$^-$ *thr-1 leuB6 proA2 hisG4 argE3 thi-1 supE44 rpsL31* Δ(*recA-srlR306*)::Tn*10* = Δ*recA306*] (both from A. J. Clark's collection) were used in bacterial conjugation experiments. Strain GY7109 [F$^-$ λ*C1 ind-1 sfiA*::*lacZ* Δ(*pro-lac*) Δ*recA306*] (R. Devoret's collection) was used for the SOS induction assay. Strain JC11001 (the same as JC10289 but *recA200 recB21 recC22 sbcB15 leu*$^+$ *metB*; A. J. Clark's collection) was used as the source of the *recA200* mutation.

Plasmid pUC19-recA1.1 was constructed by inserting a *Sac*II-*Nde*I DNA fragment, about 1,360 bp long and carrying a wild-type *recA* gene and its promoter, into the *Hin*dII site of a pUC19 vector. This plasmid was used for site-directed mutagenesis of the *recA* gene. Plasmids were maintained in strains carrying the Δ*recA306* deletion by using 50 μg of ampicillin per ml.

PCR-based site-directed mutagenesis of the *recA* gene. All new *recA* mutations were constructed by a method developed by Nelson and Long (15). The method used employs four synthetic oligonucleotide primers (A, B, C, and D) in a three-step procedure (for details, see Fig. 1 in reference 15). The structures of the primers are presented in Table 1. The rationale for the design of the primers was as follows. The type A primers contain the desired single-base mismatch (or a degenerated base as a variant) to direct mutagenesis. The other three primers were constructed to allow selective amplification of the mutated *recA* sequence with *Thermus thermophilus* polymerase. Primer B is a 40-mer hybrid oligonucleotide, 20 nucleotides of which at the 3′ terminus were designed to be identical to the pUC19 sequence located to the right of the *recA* gene in plasmid pUC19-recA1.1, whereas the other 20 nucleotides were chosen as an arbitrary nucleotide sequence. The latter was identical to the sequence of primer D. Primer C was the sequence of pUC19 located to the left of the *recA* gene in plasmid pUC19-recA1.1.

The three-step mutagenesis procedure was as follows. In step 1, hybrid primer B and mutation primer A were used to amplify a DNA fragment carrying the designed mutation. One of the strands of this newborn amplicon bearing the mutation near its 3′ end was used in step 2 as a primer to elongate this strand by *Tth* polymerization on the pUC19-recA1.1 DNA matrix. The product of the reaction was used as a template for amplification (step 3) with primers D and C that resulted in the formation of an amplicon with a selected mutation.

Mutated *recA* genes were cut out by using restriction endonucleases *Kpn*I and *Hin*dIII and integrated into plasmid pUC19 through the same restriction sites. The fragments of the *recA* gene carrying the designed mutations were sequenced by the Sanger dideoxy-mediated chain termination method (20) with T7 DNA polymerase (T7 Sequenase Kit; Pharmacia) and appropriate oligonucleotide primers. The sequenced fragments carrying identified mutations were recloned into plasmid pUC19-recA1.1 by substitution of a homologous fragment. The following restriction sites were used for recloning: *Bst*EII for the *recA2183* mutation, *Hin*dII for the *recA2223* mutation, and *Eco*RI-*Hin*dIII for all other mutations (*recA2277*, *recA2278*, *recA2278-5*, *recA2283E*, and *recA2284*).

Amplification procedure. For all of the mutagenesis steps, the reaction mixture contained 50 mM Tris-HCl (pH 8.6), 6 mM $MgCl_2$, 12 mM $(NH_4)_2SO_4$, 100 mg of bovine serum albumin per ml, 50 pmol of each primer, 200 mM each deoxynucleoside triphosphate, 1 pmol of template DNA, and 2.5 U of *T. thermophilus* polymerase (Biomaster, Moscow, Russia). In step 1, the amplicon was synthesized by 30 cycles of amplification. Each cycle consisted of DNA melting (1 min at 94°C), DNA annealing (1 min at 37°C), and DNA synthesis (1.5 min at 72°C). This amplicon was purified from 1.5% NuSieve agarose (FMC) after gel electrophoresis (Prep-A Gene Kit; Bio-Rad). Step 2 consisted of three cycles with the following temperature variations: 2 min at 94°C, 8 min at 50°C, and 3 min at 72°C. The reaction mixture contained 1 pmol of amplicon 1, 1 pmol of plasmid pUC19-recA1.1, and all other necessary components. At step 3, primers C and D were added to the same mixture and the DNA was amplified for 30 cycles programmed for 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C. This amplicon was purified in the same manner as described for step 1.

Detection of the cellular level of RecA proteins. Each JC10289 strain carrying on the pUC19 plasmid the wild-type or a mutant *recA* allele was grown in LB medium to the early log phase at 37°C and then additionally to the mid-log phase at 42°C. A cell pellet containing 2×10^7 cells was lysed by boiling with sodium dodecyl sulfate, electrophoresed through sodium dodecyl sulfate–12% polyacrylamide gels, and subjected to Western blotting (immunoblotting) with polyclonal rabbit antibodies to the *E. coli* RecA protein by using standard methods (20). Primary antibody binding was visualized with secondary antibodies coupled to horseradish peroxidase (DAKO-Immunoglobulins). The blots were then stained with diaminobenzidine (Sigma) and photographed. The negatives were scanned with a soft laser scanning densitometer (ScanJet IIp; Hewlett-Packard), and the relative amount of stain presented in each RecA protein spot was computed by using an original integrator program developed in our division.

Assay for homologous recombination at 32 and 42°C. The recombination proficiency of JC10289 (Δ*recA306*) cells carrying *recA*$^+$ or different *recA* mutant alleles on plasmid pUC19 was determined as the yield of Leu$^+$ Str^r recombinants formed at 32 or 42°C after mating of KL227 donors with JC10289 recipients. The donors and recipients were grown in LB medium (20) at 32 or 42°C to the mid-log phase and were mixed for conjugation at a ratio of 1 donor to 10 recipients at 37 or 42°C, respectively. We used 37°C instead of 32°C during conjugation because the process was found to be very inefficient at 32°C. Mating occurred for 90 min with gentle shaking. After vigorous vortexing, the 37 and 42°C mixtures were plated on M9 minimal agar (suitable for selection of Leu$^+$ Str^r recombinants) which was preheated at 32 and 42°C, respectively. The former plates were incubated overnight at 32°C. The latter plates were incubated for 3 h at 42°C and then overnight at 37°C. We changed the temperature to prevent loss of plasmid pUC19-recA1.1 or its derivates from JC10289 exconjugants. Besides, 3 h of exposure to 42°C was enough to complete the recombination process as monitored by the RecBCD recombination pathway (2).

UV repair assay at 32 and 42°C. The repair proficiency of JC10289 (Δ*recA306*) cultures, determined by plasmid pUC19 carrying *recA*$^+$ or different *recA* mutant alleles, was determined. Each culture was grown in LB medium to the mid-log phase at 32 or 42°C. After centrifugation, the cells were resuspended in saline (0.9%) at a concentration of about 10^7/ml. A 1-ml portion placed in a petri dish was exposed to various fluences of UV light generated by a germicidal mercury vapor lamp under emission conditions calibrated as 30 J/m^2/min. Survivors were determined by plating appropriate dilutions on LB agar and incubating them overnight at 32 or 42°C, respectively.

Assay for SOS-induced gene expression at 32 or 42°C. The comparative abilities of different *recA* mutations to induce an SOS response were tested in strain GY7109 (Δ*recA306 sfiA*::*lacZ*) carrying plasmid pUC19-recA1.1 and its mutant derivates. The cultures were grown to the mid-log phase in LB medium at 32 or 42°C and treated with nalidixic acid (50 μg/ml) at the same temperature. The SOS response was determined by measurement of β-galactosidase synthesis within the cells at different times after the start of the treatment.

RESULTS AND DISCUSSION

Cloning and sequencing of the *recA200* allele. The *recA200* mutation was cloned into vector pUC19 by insertion of a *Sac*II-*Nde*I DNA fragment from strain JC11001 (*recA200*) into the *Hin*dII site of the pUC19 polylinker. Cloning was confirmed by restriction analysis of constructed plasmid pUC19-recA200 (see the upper line in Fig. 1), as well as the expected phenotypic characteristic of strain JC10289 (Δ*recA306*)/pUC19-

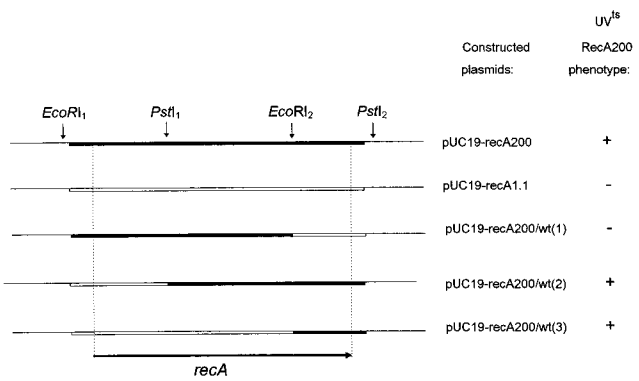


FIG. 1. Schemes of pUC19-recA derivatives constructed for localization of *recA200* mutations. The vertical arrows show restriction endonuclease sites used in the analysis. In plasmid pUC19-recA200, a black bar shows the DNA fragment (carrying the *recA200* gene) integrated into plasmid pUC19. The latter is shown by a thin line. In plasmid pUC19-recA1.1, an empty bar shows the DNA fragment (carrying the wild-type *recA* gene) integrated into plasmid pUC19. In the other plasmids, combinations of black and empty bars show different portions of DNA fragments resulting in different hybrid *recA200-recAwt* genes. The vertical dotted lines show the position of the *recA* gene. The latter is represented by the horizontal arrow.

recA200. The latter showed high sensitivity to UV light at 42°C (UV^{ts} phenotype [14]).

To localize the allele(s) determining the UV^{ts} phenotype, two hybrid *recA*$^+$*-recA200* genes were constructed by replacement of *Eco*RI or *Pst*I fragments of plasmid pUC19-recA1.1 with the same fragments from plasmid pUC19-recA200 (Fig. 1). The desired phenotype was found to be connected with the *Pst*I fragment from the *recA200* locus [the pUC19-recA200/wt(2) plasmid in Fig. 1]. The third *recA*$^+$*-recA200* hybrid, constructed by insertion of the *Eco*RI fragment of pUC19-recA1.1 into plasmid pUC19-recA200, confirmed that at least one mutant allele responsible for the UV^{ts} phenotype is located in the portion of the *recA200* gene carrying the DNA fragment flanked by *Eco*RI_2 and *Pst*I_2 sites [plasmid pUC19-recA200/wt(3)]. In fact, the sequence of the fragment revealed a nucleotide change in codon 283 of the *recA* gene (ctg283ccg) that generates amino acid substitution L-283→P in the mutant RecA protein. The mutant allele was called *recA2283*. The phenotypic properties of the mutant were compared with those of other mutants constructed in this study.

Comparison of steady-state cellular levels of RecA proteins produced by mutant and wild-type *recA* genes. To be sure that phenotypic properties of a mutant RecA protein cannot be explained by lowering of the cellular level of the protein, it was necessary to assay the level of each mutant protein relative to that of a wild-type protein. The experiment was carried out as described in Materials and Methods. Because some of the mutant proteins were expected to be thermosensitive, all JC10289 (Δ*recA306*) cells carrying *recA*$^+$ or mutant *recA* alleles on plasmid pUC19 were grown at 42°C for at least 3 generations before the cells were lysed.

The results of immunoblotting of different RecA proteins are presented in Fig. 2. The amount of mutant RecA proteins detected after lysis of 2×10^7 cells from each culture was estimated in relative units compared with wild-type RecA. It is easy to see that the steady-state cellular level of any of the mutant RecA proteins analyzed was not altered enough to explain the mutant properties described below.

FT mutations. Two FT mutations have been described earlier (6, 8, 14). One, *recA444*, generates the change of a very conservative Val (as judged by the multisequence alignment of eubacterial RecA protein analogs [19], the list of which was extended by other RecA sequences available from GenBank) to Met that results in a rather "soft substitution" (V-246→M) because both amino acid residues belong to a functionally related class of hydrophobic nonpolar (HNP) amino acids. The other mutation, *recA2283*, discussed above, generates the change L-283→P in the RecA2283 protein. According to the previously mentioned alignment, Leu in position 283 of the RecA protein is nonconservative. That is probably why the substitution to structurally distinct but functionally related Pro was necessary to produce the FT phenotype. Indeed, the introduction of Pro with its rigid conformation can reduce the structural flexibility of the RecA protein in this position.

Taking into account these observations, we formulated the strategy of searching for other FT mutations. A substituted amino acid residue must be functionally related to the original one, and it should be chosen by considering the natural conservation of the original.

In principle, our data support the correctness of the strategy. Table 2 summarizes the properties of new mutants analyzed in the manner described above for the description of both *recA444* and *recA2283*. In addition to *recA2283*, two single mutations *recA2223* and *recA2278*, and one double mutation, *recA2278-5*, produce the desired FT phenotype. The latter three mutations produced, respectively, amino acid substitutions L-223→M, G-278→P, and G-278→T plus V-275→F. With one exception (G-278→P), all of the amino acids participating in the substitutions are functionally related; they belong to either an HNP (F, L, M, P, and V amino acids) or a polar uncharged (POU) (G and T amino acids) group. The G-278→P change implies functional conversion of POU amino acid residue Gly to Pro, which is of the HNP type. However, such a functional conversion (POU to HNP) has been found in the RecA multisequence alignment as a possible variant for a Gly-278→Ala substitution (Table 2). Thus, the substitutions resulting in FT phenotypes can be referred to as soft.

Quite another picture was found in the case of unconditional mutations *recA2283E*, *recA2284*, *recA2277*, and *recA2183*, which generate the substitutions L-283→E, I-284→D, L-277→N, and K-183→M, respectively. All of them are connected with strong functional amino acid changes from HNP to negatively charged, from HNP to POU, and from positively charged to HNP.

Below, we describe in detail individual mutants, as well as the reasons why some amino acid residues were chosen for the construction of given substitutions.

αH-β9 region. The L-283→P substitution is located in the nonstructural loop between the αH and β9 structures (23). This loop and some amino acid residues located in the β9 strand are characterized by a high, and probably well-balanced, concentration of charged residues (Fig. 3A). The sequence is ^{280}KEKLIEK^{286} (19), where K is a positively charged Lys res-

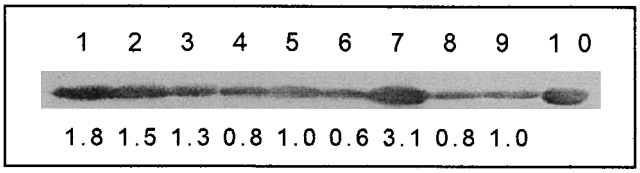


FIG. 2. Detection of mutant RecA proteins by Western blotting with antiserum raised against the *E. coli* RecA protein. RecA proteins from extracts of JC10289 cells containing plasmid pUC19 carrying *recA2283*, *recA2223*, *recA2183*, *recA2277*, *recA2283E*, *recA2284*, *recA2278-5*, *recA2278*, and *recA*$^+$ were present in lanes 1 to 9, respectively. Purified protein RecA was in lane 10. The numbers below the lanes show the amounts of RecA protein in relative units.

TABLE 2. Properties of RecA mutant proteins analyzed

recA allele	Amino acid substitution(s) generated	Functional conversion(s) generated[a]	Conservation characteristic of original residue[b]	Phenotype revealed[c]		
				Rec	UV	SOS
2283	L-283→P	HNP→HNP	N/C (V, I, M, F)	TS	TS	TS
2283E	L-283→E	HNP→NC	N/C	+	+	+
2284	I-284→D	HNP→NC	N/C (V)	−	−	−
2277	L-277→N	HNP→POU	VC	+/−	+/−	+/−
2278	G-278→P	POU→HNP	C (A)	TS	TS+/−	TS
2278-5	G-278→T +	POU→POU+				
	V-275→F	HNP→HNP	C, N/C	TS	+/−	TS+/−
2223	L-223→M	HNP→HNP	VC	TS+/−	TS+/−	TS
2183	K-183→M	PC→HNP	N/C (S, N, A)	−	−	−

[a] Amino acid functional classifications: HNP, hydrophobic nonpolar; NC, negatively charged; PC, positively charged; POU, polar uncharged.
[b] The conservation characteristic is based on the RecA protein multisequence alignment taken from reference 19 and enlarged by new data from GenBank. NC, nonconservative amino acid residue; VC, very conservative residue; C, conservative residue. Letters in parentheses designate the residues found in the alignment.
[c] Symbols: +, −, and +/− ability, inability, and moderate ability, respectively, to carry out recombination (Rec), repair of UV damage (UV), and the SOS response (SOS); TS and TS+/−, respectively, strong and moderate functional inability at 42 versus 32°C.

idue, E is a negatively charged Glu, and the residue of interest, Leu, lies in the middle. Since the L-283→P substitution produced the FT phenotype, we also decided to change the charge balance of the sequence described above by introduction of an additional negative charge with substitution L-283→E or I-284→D.

Comparative analysis of the recombination proficiencies at 32 and 42°C of these mutants and both necessary controls, *rec*$^+$ and Δ*recA306*, is presented in Table 3. Proficiency was estimated as the yield of Leu$^+$ Str^r recombinants in crosses between KL227 donors and JC10289 Δ*recA306* recipients, the recombination capability of which was determined by a family of pUC19-recA plasmids carrying different *recA* alleles. The analysis showed that mutations *recA2283* (resulting in the substitution L-283→P), *recA2283E* (L-283→E), and *recA2284* (I-284→D) generated strong Rec^{ts} (deficient at 42°C), Rec$^+$, and Rec$^-$ phenotypes, respectively.

Fig. 4A and B depicts the repair characteristics of the same three *recA* alleles assayed at 32 and 42°C by UV survival of strain JC10289 carrying appropriate pUC19-recA plasmids. These *recA* mutations show, respectively, inhibition of UV repair at 42 versus 32°C (i.e., the UV^{ts} phenotype), a wild-type level of repair proficiency (UV$^+$), and strong repair deficiency at both temperatures (UV$^-$).

The abilities of different *recA* alleles to complement the SOS response defect of the Δ*recA306* mutation were tested with strain GY7107 (Δ*recA306 sfiA*::*lacZ*) carrying plasmid pUC19-recA with the assayed allele. The data in Fig. 5A and B compare the levels of β-galactosidase production caused by the SOS-inducible *sfiA*::*lacZ* fusion gene when the cells were treated with nalidixic acid for different times at 32 or 42°C.

Note that the level of β-galactosidase expression was found to be lower for all *recA* alleles, including *recA*$^+$, at 42°C than at 32°C. The simplest explanation of this unexpected observation is that there is more loss of pUC19-recA plasmids from strain GY7107 at the higher temperature. Because this was a comparative analysis and all of the data were normalizing to control *recA*$^+$ dependence, the complication discussed above does not change our final qualitative conclusions. Thus, the SOS proficiencies of *recA2283*, *recA2283E*, and *recA2284* mutants can be described as SOS^{ts}, SOS$^+$, and SOS$^-$, respectively.

Taken together (Table 2), the recombination, repair, and SOS characteristics of these three mutants show that our attempt to generate an FT phenotype due to a change in charge balance in the loop between the αH and β9 structures was successful in only one of the two neighboring positions. Both Leu-283 and Ile-284 can be referred to as nonconserved residues. However, the former belongs to the loop, whereas the latter is involved in the β9-strand structure (Fig. 3A). In addition, the amino acid residues have different orientations in the RecA monomer three-dimensional structure that can reflect their different participation in interactions within the RecA protein.

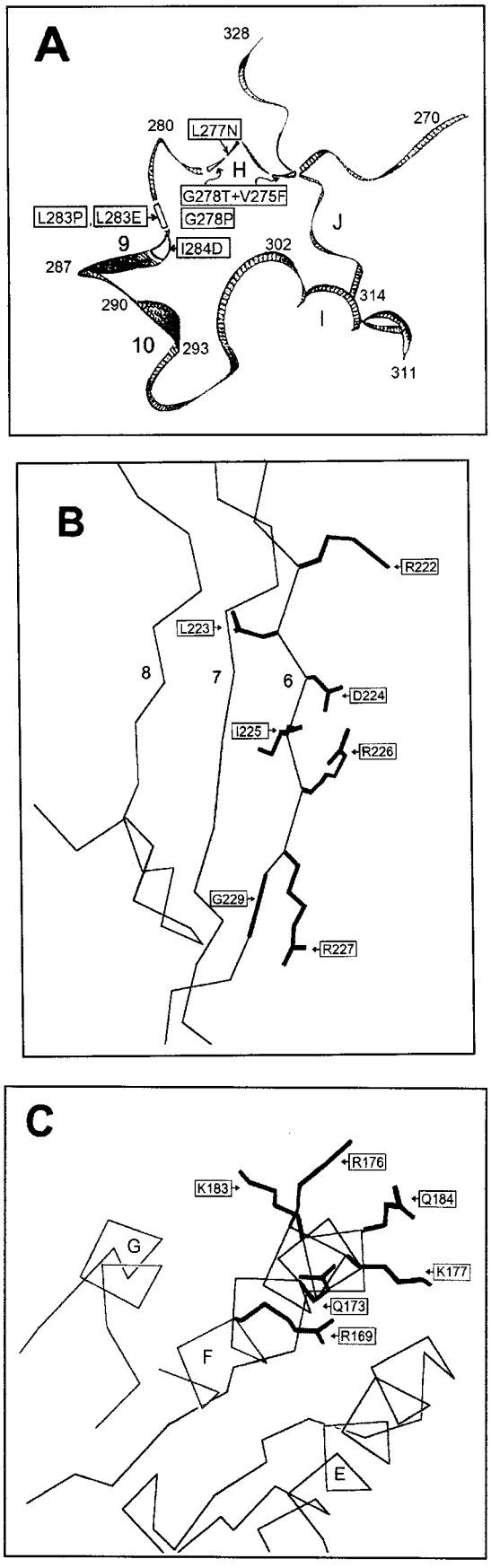
Three other substitutions were designed within α-helix H (Fig. 3A). The Leu-277 residue was chosen as an object of alterations because it is a very conservative. Gly-278 is also a conserved residue. Besides, the minimal steric hindrance of the glycine side chain allows much more structural flexibility than other amino acids. From this point of view, Gly is not a suitable constituent for an α-helix structure although two Gly residues are represented in α-helix H of the *E. coli* RecA protein.

The L-277→N substitution was designed to produce a functional conversion of the residue in this position. Both substitutions G-278→P and G-278→T were intended to modify α helicity by a much more rigid and a more rigid amino acid change, respectively. In additional to the latter, the V-275→F substitution arose occasionally and thus the *recA2278-5* double mutant appears to have been formed.

The comparative quantitative data on the recombination, repair, and SOS response properties of *recA2277*, *recA2278*, and *recA2278-5* mutants at 32 and 42°C are represented in Table 3, Fig. 4A and B, and Fig. 5A and B, respectively. Their qualitative characteristics are summarized in Table 2. The L-277→N substitution resulted in a moderate deficiency in all three analyzed genetic phenotypes that can be symbolically described as Rec$^{+/-}$, UV$^{+/-}$, and SOS$^{+/-}$. The alteration G-278→P strongly inhibited recombination and SOS induction abilities at 42°C, whereas UV survival ability was only moderatively suppressed at this temperature. Thus, the summary phenotype of the *recA2277* mutant is Rec^{ts} UV$^{ts+/-}$ SOS^{ts}.

The characteristics of the *recA2278-5* double mutant appeared to be most unusual. This mutant demonstrated the strongest recombination deficiency at 42°C of all of mutants analyzed, whereas two other phenotypes, which were expected to be internally connected with the former, were different. In fact, SOS induction was only moderately thermosensitive at 42°C, whereas UV repair was moderately deficient without any expressed thermosensitivity. In sum, the mutant can be described as Rec^{ts} UV$^{+/-}$ SOS$^{ts+/-}$.

This unusual phenotype stimulated our interest in the biochemical properties of the protein encoded by the double mutation. The RecA2278-5 protein showed polyfunctional defi-



ciencies, including a reduced ability to bind single- or double-stranded DNA at 42°C and inhibition of DNA-free RecA multimerization at 42 versus 32°C. One additional deficiency, decreased binding to ATP, was also observed at 42°C (1). At least two of these defects, the double-stranded DNA-binding and RecA-RecA multimerization activities, have been described earlier in connection with inactivation of α helices I and J of the C-terminal domain of the RecA protein (7). It became clear that although the C-terminal domain protrudes from an active RecA filament structure, its integrity is important for basic functional activities of the RecA protein.

RecA2278-5 was referred to as a new class of RecA mutant proteins whose functional activities are ligand dependent. Indeed, all three mentioned thermosensitive deficiencies of RecA2278-5 were found only when the protein was preheated at 42°C before its binding to ATP. Preliminary binding of the protein to ATP restores its normal functioning at 42°C (1).

β6 strand. The β6 strand and seven other β strands (three of which are shown in Fig. 3B) form the β-sheet structure of the central RecA protein domain (23). This strand consists of the following seven amino acid residues: ^{221}Val-Arg-Leu-Asp-Ile-Arg-Arg^{227}. The most interesting feature of β6 is a high concentration of mutations that display a discriminating protease activity towards some of their usual protein substrates. In fact, the *recA1735* mutation, generating a R-222→C substitution, induces prophage ϕ80 poorly (4). The RecA142 protein, containing an I-225→K change, together with biochemical defects essential to its recombination activity (9), was found to be deficient in LexA protein cleavage but is proficient in λ repressor cleavage (18). A close neighbor of the β6 strand, the G-229→S substitution generated by the *recA91* mutation, results in inhibition in vivo of ϕ80 prophage induction without any effect on λ prophage induction (16). The RecA1734 protein, with the R-243→L substitution (the latter is located in a β7 strand and belongs to the same space region of the RecA monomer three-dimensional structure [23]), is capable of mediating the cleavage of phage λ and LexA repressors but defective in cleavage of the ϕ80 repressor or UmuD protein (4).

Thus, the β6 strand and some neighboring structures, especially after RecA protein multimerization in an active filament, can form the protein region responsible for recognition and allosteric interaction with SOS or phage repressors to stimulate their autodigestion and subsequent SOS function induction (11). It seemed reasonable to try to obtain an FT mutant by altering this special region.

The Leu-223 residue was chosen for mutagenesis because of its strong conservation within the RecA protein superfamily. The substitution L-223→M, caused by mutation *recA2223*, shows unusual, although expected, phenotypic characteristics. The mutant showed a moderate temperature-dependent decrease of recombination (compare the yields of Leu$^+$ Str^r recombinants at 32 and 42°C in Table 3) and repair (compare the 32 and 42°C UV survival curves in Fig. 4A and B) capabilities. It was found to be strictly thermosensitive in the SOS

FIG. 3. (A, B, and C) Fragments of the three-dimensional RecA protein structure proposed by Story et al. (23). The coordinate numbers and the labeling of secondary structure elements are according to the original data (23). (A) The C-terminal domain drawn by using the Ribbon computer program. α-helices H, I, and J and β-strands 9 and 10 are shown. The arrows indicate the positions of amino acid substitutions (boxed designations) in different structural elements. (B) The fragment of the major RecA domain including β-strands 6, 7, and 8 drawn by using the Kinemage program. The thick lines indicate the positions of amino acids (boxed designations). (C) The fragment of the major RecA protein domain including α-helices E, F, and G visualized by the Kinemage program. Amino acid designations are boxed.

TABLE 3. Recombination proficiencies determined by different *recA* mutations at 32 and 42°C

Temp (°C)	Relative yield of Leu$^+$ Str^r recombinants[a]								
	Wild type[b]	*recA2283*	*recA2283E*	*recA2284*	*recA2278*	*recA2278-5*	*recA2277*	*recA2223*	*recA2183*
32	1.00 (6.1)	0.26	0.82	0.002	0.62	0.66	0.50	0.66	0.003
42	1.00 (4.2)	0.04			0.06	0.01	0.59	0.30	

[a] The recombinants were found in crosses between donor KL227 and recipient JC10289 (Δ*recA306*), the recombination proficiency of which was determined by pUC19-recA plasmids carrying different *recA* alleles.
[b] The values in parentheses show the yield of recombinants as a percentage of the donor cell concentration in the initial conjugational mixture.

response that was inhibited at 42°C (Fig. 5A and B). In the terms used above, this phenotype can be designated Rec$^{ts+/-}$ UV$^{ts+/-}$ SOS^{ts} (Table 2). It predicts special biochemical properties for the RecA2223 protein with a thermodependent SOS repressor cleavage capability.

α helix F. A primary idea about the possible role of basic residue distribution in an α-helix polypeptide for recognition of structural features of DNA comes from the studies of Zlotnick and Brenner (25) on a DNA-binding region of the *E. coli* RecA protein identified as an α-helix A (23). The idea was generalized by Churchill and Travers (3) in their description of some protein motifs capable of forming a structural framework in which positively charged residues can be arranged on one face of the motif structure to bind to a particular DNA structure.

In full accordance with that idea, α-helix F of the RecA central domain was chosen by us as a candidate for another site of RecA-DNA interaction (Fig. 3C). In fact, this long structure, including 21 residues from Gly-165 to Ser-185, contains a set of positively charged residues, of which three (Arg-169, Arg-176, and Lys-177) belong to highly conserved residues and another (Lys-183) can be referred to as a conserved residue.

The following observations are in agreement with the prediction discussed above. On one hand, the loss of a positive charge by the R-169→H substitution in the RecA423 protein results in, among some other properties, a decrease in protein binding to single-stranded DNA (22). On the other hand, the introduction of a new positive charge by the Q-184→K amino acid change in protein RecA1202 produces enhancement of protein binding to single-stranded DNA that is consistent with such properties of the protein as a higher level of LexA repressor cleavage and its coprotease constitutive activity (24). In turn, the latter can be reduced by a neighboring K-177→Q substitution, generated by an intragenic suppressor *recA1630* mutation (13), probably because of the loss of positively charged residue Lys-177.

This is the reason why we constructed the substitution K-183→M with the loss of positively charged residue Lys-183. The phenotypic properties of the *recA2183* mutant were as follows. It appeared to be completely recombination deficient (Table 3), highly sensitive to UV light (Fig. 4), and absolutely SOS deficient (Fig. 5), thus showing the null phenotype Rec$^-$ UV$^-$ SOS$^-$ (Table 2).

This phenotype was not unexpected, because residue Lys-183 had been described as participating in interfilament interaction (13). The latter is thought to be necessary either for storage of the RecA protein supply within the cell in the form of filament bundles (23) or for promoting homologous recombination through the formation of filament bundle-like structures (13). One possibility is that the K-183→M substitution inhibits the interaction of RecA protein with DNA. This is consistent with the idea discussed above, as well as with recent photo-cross-linking experiments with bromouracil-DNA to show that Lys-183 became cross-linked to DNA (5, 14a). An-

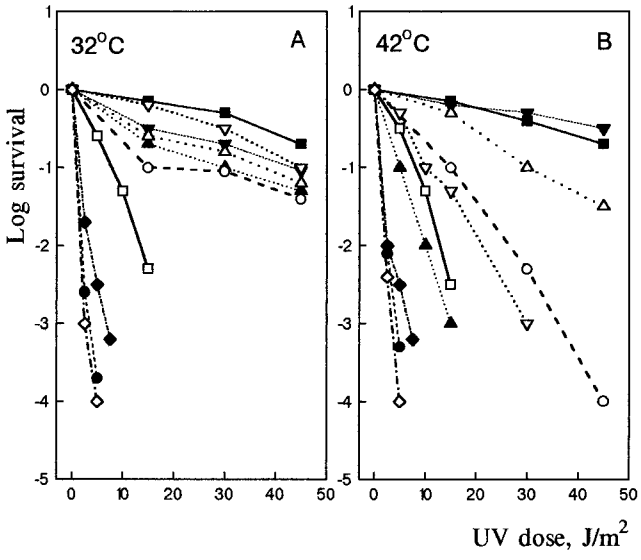


FIG. 4. (A and B) UV repair proficiencies of different *recA* mutants at 32 and 42°C. The amino acid substitutions generated by the *recA* mutations were as follows: K-183→M, *recA2183*; L-223→M, *recA2223*; L-277→N, *recA2277*; G-278→P, *recA2278*; G-278→T plus V-275→F, *recA2278-5*; L-283→P, *recA2283*; L-283→E, *recA2283E*; I-284→D, *recA2284*. Symbols: ■, *rec*$^+$; ●, *recA*$^-$; ▲, *recA2283*; ▼, *recA2283-E*; ◆, *recA2284*; □, *recA2277*; ○, *recA2278*; △, *recA2278-5*; ▽, *recA2223*; ◇, *recA2183*.

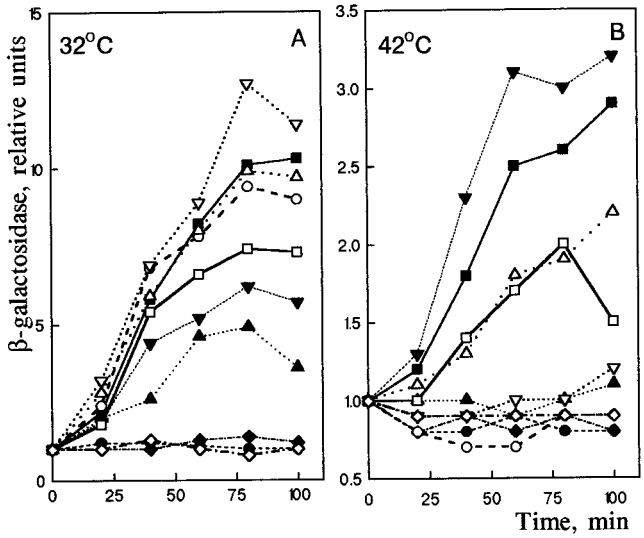


FIG. 5. (A and B) SOS induction capabilities of different *recA* mutants at 32 and 42°C. For symbol definitions and the amino acid substitutions generated by the mutations, see the legend to Fig. 4.

other possibility is that mutant protein RecA2183 is deficient in an important position for interfilament contact that might be one of the necessary steps in the strand exchange reaction in vivo. Biochemical analysis of the protein is necessary to make a choice between these possibilities.

Thus, we constructed seven and compared the phenotypic characteristics caused by eight *recA* mutations located in three different regions of the RecA protein structure. According to the properties described, all of the mutants fall into four classes: an FT class (*recA2223*, *recA2278*, *recA2278-5*, and *recA2283*), a moderately deficient class (*recA2277*), a null phenotype class (*recA2284* and *recA2183*), and a class without obvious phenotypic changes (*recA2283E*). Biochemical analysis of some of the mutant proteins is in progress.

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