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C-terminal Deletions of the *Escherichia coli* RecA Protein

CHARACTERIZATION OF *IN VIVO* AND *IN VITRO* EFFECTS*

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A set of C-terminal deletion mutants of the RecA protein of *Escherichia coli*, progressively removing 6, 13, 17, and 25 amino acid residues, has been generated, expressed, and purified. *In vivo*, the deletion of 13 to 17 C-terminal residues results in increased sensitivity to mitomycin C. *In vitro*, the deletions enhance binding to duplex DNA as previously observed. We demonstrate that much of this enhancement involves the deletion of residues between positions 339 and 346. In addition, the C-terminal deletions cause a substantial upward shift in the pH-reaction profile of DNA strand exchange reactions. The C-terminal deletions of more than 13 amino acid residues result in strong inhibition of DNA strand exchange below pH 7, where the wild-type protein promotes a proficient reaction. However, at the same time, the deletion of 13–17 C-terminal residues eliminates the reduction in DNA strand exchange seen with the wild-type protein at pH values between 7.5 and 9. The results suggest the existence of extensive interactions, possibly involving multiple salt bridges, between the C terminus and other parts of the protein. These interactions affect the pK_a of key groups involved in DNA strand exchange as well as the direct binding of RecA protein to duplex DNA.

The bacterial RecA protein plays a central role in the processes of homologous DNA recombination and DNA repair. RecA is a DNA-dependent ATPase that catalyzes an *in vitro* strand exchange reaction between single-stranded DNA and homologous double-stranded DNA molecules. The RecA protein of *Escherichia coli* consists of 352 amino acid residues. The three-dimensional structures of both the RecA protein alone and complexed with inhibitory ADP cofactor have been determined (1, 2). There is a central core domain and two smaller N- and C-terminal domains. The core domain contains the nucleotide binding site and the putative DNA binding site(s). This domain is highly conserved among bacterial and eukaryotic RecA homologs (3–6) and exhibits sequence and/or structural homology with a range of proteins including DNA helicases (7), DNA pumps (8), the F1-ATPase (9), and adenosylcobinamide kinase/adenosylcobinamide phosphate guanylyltransferase (10). The amino-terminal domain of RecA is involved in monomer-monomer interactions (1, 3). The carboxyl-terminal domain of RecA protein consists of residues 270–352, the last 24 of which are disordered in the apoenzyme crystal structure (1, 2).

The present study focuses on the C-terminal 25 amino acid residues, which we will refer to as the C terminus (as opposed to the entire domain). Over half of these terminal 25 residues have side chains that are either negatively charged (7 of the last 17 are Glu or Asp residues) or contain hydroxyl groups (six Ser or Thr residues) (Fig. 1). Positively charged amino acid side chains are absent. Other ssDNA[1]-binding proteins such as single-stranded binding protein (SSB) of *E. coli* and the gene 32 protein of phage T4 also have highly negatively charged C-terminal regions. Upon C-terminal deletion to remove these negative charges, SSB and the gene 32 protein show increased dsDNA affinities relative to the intact proteins (11, 12).

Primary structure provides few clues to the function of the RecA C terminus. Sequence conservation in this part of the protein is quite limited even when comparisons are limited to other bacterial RecA proteins. The major feature of the primary structure in the *E. coli* protein is the preponderance of negatively charged residues in this region. This feature is found in most but not all other bacterial RecA sequences. A few RecA proteins, notably from *Bacteroides* and *Mycoplasma* species, lack this protein segment altogether (4). In a few other species, particularly *Streptomyces*, the C terminus is lengthened and exhibits a preponderance of positively charged residues (4).

In addition to a general lack of structural information about the RecA C terminus, there has been little indication that this part of the protein has functional significance. Several C-terminal deletion mutants of the *E. coli* RecA protein have been characterized. Ogawa and colleagues (13) characterized RecAΔC25 (RecA5327), and Benedict and Kowalczykowski (14) described a RecA mutant in which a fragment of the protein, about 15% of the of the RecA polypeptide, was missing from the C terminus. The primary reported effect of these deletions was a faster nucleation, leading to filament formation on dsDNA, reducing the telltale lag in dsDNA-dependent ATP hydrolysis that is well documented with wild-type RecA (15, 16). It was proposed that the negatively charged C terminus of RecA regulates the binding of RecA to dsDNA by electrostatically repelling the phosphate backbone of the DNA (13, 14). Both C-terminal deletion mutants were shown to be proficient in the key RecA protein reaction of DNA pairing, and the RecAΔC25 protein promoted significant levels of final product formation

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[1] The abbreviations used are: ssDNA, single-stranded DNA; SSB, single-stranded binding protein; dsDNA, double-stranded DNA; ATPγS, adenosine 5′-*O*-(thiotriphosphate); DTT, dithiothreitol; TAPS, 3-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-1-propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; PEP, phosphoenolpyruvate; ORF, open reading frame; WT, wild type.

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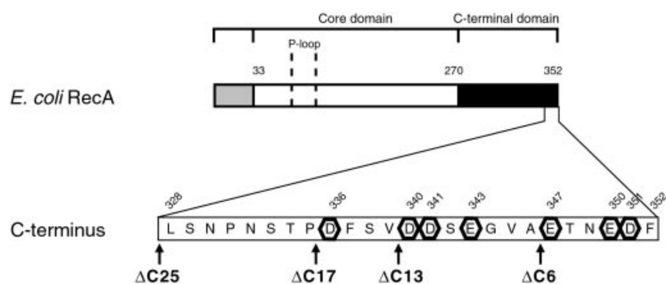

FIG. 1. **The C terminus of the *E. coli* RecA protein.** The core domain (amino acids 33–270), highly conserved among bacterial RecA proteins, is shown in *white*. This region includes the P-loop motif for ATP binding. The *shaded* and *black regions* of the sequence correspond to the N-terminal and C-terminal domains, respectively. The primary structure of the C-terminal 25 amino acids of the RecA protein (residues 328–352) is diagrammed below the linear sequence. These residues are disordered in the crystal structure of Story *et al.* (1). The *hexagons* highlight the high concentration of negatively charged amino acids in this region. The *arrows* indicate points of truncation in the deletion mutants: RecAΔC6, RecAΔC13, RecAΔC17, and RecAΔC25.

during DNA strand exchange under at least two sets of conditions.

Somewhat shorter C-terminal deletions of RecA protein have also been constructed and characterized. A 17-residue C-terminal deletion mutant was shown not to affect UV resistance, induction of the SOS response, or Weigle reactivation (17). The same study showed that this mutant had a minimal effect on recombination when expressed by itself. A small effect on conjugational recombination was observed only when the wild-type and mutant proteins were both present *in vivo* (17). The removal of about 18 residues from the C terminus resulted in a substantial conformational difference in RecA filaments bound to dsDNA, as observed in three-dimensional reconstructions of electron microscopy images (18). The biochemistry of these C-terminal deletion mutants was not explored.

We have initiated an effort to explore the function of the C-terminal 25 amino acid residues of RecA protein in more detail. To better define what parts of this segment are involved in activity changes, we have constructed a set of C-terminal deletion mutations, removing 6, 13, and 17 amino acid residues. These remove 3, 6, and 7 of the negatively charged amino acid residues, respectively (Fig. 1). The RecA protein mutant with 25 amino acids removed from the C terminus was also included in part to provide a reference point with which to compare our results with those previously published by Ogawa and co-workers (13). The first study examines the effects of these mutations on cell survival and on fundamental *in vitro* properties of RecA protein.

EXPERIMENTAL PROCEDURES

Enzymes and Biochemicals—*E. coli* SSB was purified as described (19). The concentration of the purified SSB protein was determined from the absorbance at 280 nm using the extinction coefficient of 2.83×10^4 M^{-1} cm^{-1} (20). SSB was also purchased from Sigma. Unless otherwise noted, all reagents were purchased from Fisher and were of the highest grade available. *Eco*RI and *Hin*dIII restriction endonucleases were purchased from New England Biolabs. ATPγS was purchased from Roche Molecular Biochemicals. DTT and TAPS were obtained from Research Organics. CHES, MES, HEPES, lysozyme, phosphoenolpyruvate (PEP), pyruvate kinase, ATP, polyethyleneimine, bromphenol blue, mitomycin C, and NADH were purchased from Sigma. *Xho*I restriction endonuclease and DEAE-Sepharose Fast Flow resin were purchased from Amersham Biosciences. Hydroxyapatite resin was obtained from Bio-Rad. Isopropyl-1-thio-β-D-galactopyranoside was purchased from BioVectra.

Buffers and Media—P buffer contained 20 mM potassium phosphate (pH 6.8), 1 mM DTT, 0.1 mM EDTA, and 10% (w/v) glycerol. R buffer contained 20 mM Tris-HCl (80% cation, pH 7.5), 1 mM DTT, 0.1 mM EDTA, and 10% (w/v) glycerol. TAE buffer contained 40 mM Tris-OAc (80% cation) and 1 mM EDTA. Luria-Burtani medium (LB broth) is 10 g/liter trypone, 5 g/liter yeast extract, and 10 g/liter NaCl, with pH adjusted to 7.0. M9 minimal medium is 12.8 g/liter $Na_2HPO_4·7H_2O$, 3 g/liter KH_2PO_4, 0.5 g/liter NaCl, 1.0 g/liter NH_4Cl, and 4% (w/v) glucose.

DNA Substrates—Bacteriophage ϕX174 circular single-stranded DNA (virion) was purchased from New England Biolabs. ϕX174 RF I supercoiled circular duplex DNA was purchased from Invitrogen. Full-length linear duplex DNA was generated by the digestion of ϕX174 RF I DNA (5386 bp) with the *Xho*I restriction endonuclease, using conditions suggested by the enzyme supplier. The digested DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), followed by ethanol precipitation. Circular single-stranded and supercoiled circular duplex DNAs from bacteriophage M13mp8 (7229 nucleotides) was prepared using previously described methods (21–23). Circular M13mp8 duplex DNA containing a single nick (nicked circular dsDNA) was generated using a previously described method (24). The concentrations of ssDNA and dsDNA were determined by absorbance at 260 nm, using 36 and 50 μg ml^{-1} $A_{260}{}^{-1}$, respectively, as conversion factors. All DNA concentrations are given in μM nucleotides.

Cloning and Overexpressing RecA Proteins—A plasmid was constructed to express the wild-type *E. coli* RecA protein under the control of the bacteriophage T7 RNA polymerase promoter. The wild-type *recA* open reading frame (ORF) was cloned in two parts. The N-terminal coding region came from the *Nco*I-*Pst*I fragment of plasmid pTRecA103, which was a gift from Kendall Knight (25). PCR was used to generate the remaining coding region of the *recA* ORF using plasmid pGE226 as a template, kindly given by George Weinstock (26). Primers were designed to amplify from the *Pst*I site to the stop codon of the *recA* ORF with the introduction of a *Hin*dIII site just downstream of the stop codon. The full-length *recA* ORF (*Nco*I-*Hin*dIII fragment) was cloned downstream of the T7 RNA polymerase promoter in the Amp^R plasmid pET21d(+) from Novagen to generate plasmid pAIR79. Both strands of the *recA* ORF were manually sequenced to ensure the integrity of the coding region.

To overexpress the wild-type RecA protein, pAIR79 was co-transformed with pT7POL26 (Kan^R) into the multiply nuclease-deficient strain STL327 (*exoI*$^-$ *exoIII*$^-$ *endoI*$^-$ *recJ*$^-$) provided by Susan Lovett. The strain is also named RDK1896 (27). Plasmid pT7POL26 codes for T7 RNA polymerase under the control of a *lac* promoter (28). Eight liters of culture were grown in LB broth to an A_{600} of 0.5, and RecA protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to 0.4 mM. Following a 3-h incubation at 37 °C, ~20 g of cells were harvested by centrifugation, flash-frozen in liquid N_2, and stored at −80 °C.

The *rec*AΔC6, *rec*AΔC13, and *rec*AΔC17 genes were constructed by oligonucleotide-directed mutagenesis using the Kunkel selection method (29). An *Eco*RI-*Hin*dIII fragment from pAIR79 coding for the C terminus region of the *rec*A ORF was cloned into the Amp^R plasmid pGEM3Zf(−) from Promega to create plasmid pAIR34. This plasmid was used as the template for the *in vitro* mutagenesis. Oligonucleotides encoding stop codons were used to introduce new translational stop sites at the appropriate positions in the C-terminal coding region. Both strands of the subcloned region were manually sequenced to ensure that no other mutations were introduced during mutagenesis. Cloning was used to swap these C-terminal coding regions into plasmid pAIR79. The plasmid containing the *recA*ΔC25 gene (pTH5327) was a gift from Tomoko Ogawa (13). The plasmids containing the mutant genes *recA*ΔC6, *recA*ΔC13, and *recA*ΔC17, designated pAIR64, -45, and -46, respectively, were each co-transformed with pT7POL26 (Kan^R) (28) into the nuclease deficient strain STL2669 (Δ*recA-srlR*)306::*Tn*10 Tet^R, *xonA2* (*exoI*$^-$) in an AB1157-derived genotypic background; a gift from Susan Lovett). Culture growth and protein induction conditions were the same as for the wild-type RecA protein.

Preparation of E. coli Strains for in Vivo Experiments—The genes encoding the wild-type RecA, RecAΔC6, RecAΔC13, and RecAΔC17 proteins were each integrated into the identical site on the chromosome (base 1899 of the *rmlD* gene of MG1655 Δ*recA* tet^S 127fRT#1/pLH29 (30) using the FLIRT system (30). FLIRT makes use of the Flp site-specific recombination system to efficiently introduce cloned DNA onto the bacterial chromosome. In each case, the proteins were expressed from the wild-type recA promoter. The plasmids used to introduce wild-type *recA*, *recA*ΔC6, *recA*ΔC13, and *recA*ΔC17 to the chromosome are designated pEAW 222, 232, 230, and 229, respectively. The wild-type *recA* promoter was added to the start of each *recA* gene by including the 200 base pairs of the DNA sequence upstream of the *recA* gene in MG1655 (a gift from George Weinstock). Multiple attempts to clone the *recA*ΔC25 gene onto the chromosome in the same manner as the others were unsuccessful.

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Sensitivity of E. coli Strains Expressing Wild-type RecA, RecAΔC6, RecAΔC13, or RecAΔC17 Proteins to UV, Mitomycin C, and Ionizing Radiation—10 ml of LB broth was inoculated with 100 μl of an overnight culture of the Δ*recA* MG1655 strain containing the wild-type *recA*, *recAΔC6*, *recAΔ13*, or *recAΔC17* genes described above and grown for 1.5 h at 37 °C. Cells were pelleted in a clinical centrifuge and washed with 5 ml of M9 minimal medium. Cells were pelleted again and resuspended in 5 ml of M9 minimal medium. Cells were diluted further in M9 to an A_{600} of 0.06 in a volume of 5 ml. Further serial 1:10 dilutions were made in M9 to optimize cell density to obtain ~30–100 colonies per experiment. The various dilutions (50 μl of each) were plated on LB agar plates. Uncovered plates were exposed to UV using a Stratalinker UV cross-linker, model 1800 (Stratagene) in a darkened room at the J/m^2 indicated in the figure. The plates were incubated overnight at 37 °C in the dark. Colonies were counted using GelExpert software (Nucleotech) and adjusted for dilutions. Mitomycin C and ionizing radiation sensitivity tests were performed as with the UV tests above except that diluted cells were plated on LB agar plates containing the indicated amount of mitomycin C or exposed to ionizing radiation in a model 30 Mark I 137 cesium irradiator (J. L. Shepard and Associates) for times necessary to achieve the indicated dose and incubated overnight in the dark.

Purification of the RecA Protein—Wild-type RecA protein was purified using modifications to previously described protocols (31, 32). All steps were carried out at 4 °C. Cell paste (20 g) was thawed and fully resuspended in 80 ml of a solution of 25% (w/v) sucrose and 250 mM Tris-HCl (80% cation, pH 7.5). Cells were lysed by a 60-min incubation with 40 ml of a 5 mg/ml solution of lysozyme in 250 mM Tris-HCl (80% cation, pH 7.5), followed by the addition of 50 ml of 25 mM EDTA, sonication, and centrifugation. The lysate was precipitated with 22 ml of 5% (w/v) polyethyleneimine, pH 7.5 (0.5% final concentration), and centrifuged. The pellet was washed with 50 ml of R buffer plus 150 mM ammonium sulfate and extracted two times with 25 ml of R buffer plus 300 mM ammonium sulfate. The protein solution was precipitated by the addition of 0.28 g of solid ammonium sulfate per ml of solution (48% saturation). The resulting pellet was washed two times with R buffer plus 2.1 M ammonium sulfate, resuspended in 50 ml of R buffer plus 50 mM KCl, and dialyzed *versus* the same. The protein was loaded onto a DEAE-Sepharose column and washed with two column volumes of R buffer plus 50 mM KCl. Flow-through peak fractions were identified by SDS-PAGE analysis, pooled, and dialyzed *versus* P buffer. Protein was then loaded onto a hydroxyapatite column, washed with two column volumes of P buffer, and eluted with a linear gradient from 20–350 mM phosphate buffer (pH 6.8) over 10 column volumes. Peak fractions were identified by SDS-PAGE analysis, pooled, concentrated with Centricon Plus-20, 10,000-dalton molecular mass cut-off concentrators (Amicon) or by ammonium sulfate precipitation and resuspension in R buffer, dialyzed into R buffer, flash-frozen in liquid N_2, and stored at −80 °C. The concentration of the wild-type RecA protein (37,842 Da) was determined from the absorbance at 280 nm using the extinction coefficient $2.23 \times 10^4\ M^{-1}\ cm^{-1}$ (33). The protein was free of detectable nuclease activities.

Purification of the RecA C-terminal Deletion Mutant Proteins—The deletion mutants RecAΔC6, RecAΔC13, RecAΔC17, and RecAΔC25 were purified using the following modifications to the wild-type RecA procedure from above. (*a*) The initial polyethyleneimine pellet was washed with R buffer plus 50 mM ammonium sulfate and extracted two times with R buffer plus 150 mM ammonium sulfate. The RecAΔC6, RecAΔC13, and RecAΔC17 proteins were precipitated with 0.31 g of solid ammonium sulfate per ml of solution (48% saturation). The RecAΔC25 protein solution was precipitated with 0.28 g of solid ammonium sulfate per ml of solution. The RecAΔC25 protein was precipitated from the supernatant fraction after centrifugation with an additional 0.113 g of solid ammonium sulfate per ml of solution (to 65% saturation). Each deletion mutant protein was resuspended in R buffer plus 300 mM KCl, dialyzed one time *versus* the same buffer and then two times *versus* R + 100 mM KCl for the RecAΔC6, RecAΔC13, and RecAΔC17 proteins and *versus* R buffer for the RecAΔC25 protein. (*b*) All deletion mutants were eluted from the DEAE-Sepharose column with a linear gradient of KCl, from 100 to 500 mM for the RecAΔC6, RecAΔC13, and RecAΔC17 proteins and from 0 to 500 mM for the RecAΔC25 protein. All proteins eluted from the DEAE-Sepharose column at ~250 mM KCl. (*c*) The calculated molecular masses of the RecAΔC6, RecAΔC13, RecAΔC17, and RecAΔC25 proteins are 36,999, 36,200, 35,680 and 34,783 Da, respectively. Each mutant protein was determined to be greater than 95% pure by SDS-PAGE (Fig. 2) and free of detectable nuclease activities. The wild-type RecA extinction coefficient was used to calculate the concentration of each mutant protein.

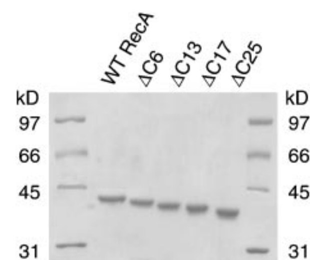

FIG. 2. **SDS-polyacrylamide gel of purified wild type and the four C-terminal deletion RecA proteins.** The purification protocols and the calculated molecular weights of each mutant can be found under "Experimental Procedures."

Electron Microscopy of Circular ssDNA-RecAΔC17 Nucleoprotein Filaments—A modified Alcian method was used to visualize RecA filaments and unreacted DNA if present. Activated grids were prepared as follows. 0.2% Alcian in 3% acetic acid was left for many days to dissolve and then centrifuged in a Millipore 0.22-μm filter unit at 5000 rpm for 40 min. 25 μl of the clear solution was diluted with 500 μl of distilled water. A carbon film (attached to an electron microscope grid) was floated on a 70-μl drop of this solution (placed on a Teflon block) for 10 min. The film was then washed by touching to a drop of water and finally floated on two 3-ml drops of water, each for 5 min. After the final wash, an empty 5-μl glass pipette was used to draw off remaining liquid from the edge of the grid by capillary action. The activated grid was then dried under a heat lamp.

Samples for electron microscopy analysis were prepared as follows. All incubations were carried out at 37 °C. wild-type RecA or RecAΔC17 protein (6.7 μM) was preincubated with 20 μM M13mp8 circular ssDNA, 25 mM Tris-OAc (80% cation) buffer, 1 mM DTT, 5% (w/v) glycerol, 3 mM potassium glutamate, and 10 mM $Mg(OAc)_2$ for 10 min. An ATP regeneration system of 10 units/ml pyruvate kinase and 5 mM PEP was also included in the preincubation. ATP and SSB protein were added to 3 mM and 2 μM, respectively, and the reaction was incubated for 10 min longer, after which ATPγS was added to 3 mM to stabilize the filaments and incubated for 5 min. An 8-μl sample of the reaction mixture described above was diluted 17-fold with 200 mM ammonium acetate, 10 mM Hepes (pH 7.0), and 10% glycerol and adsorbed to the activated carbon film for 3 min. The grid was then touched to a drop of the above buffer followed by floating on a drop of the same buffer for 1 min. The sample was stained by touching to a drop of 5% uranyl acetate followed by floating on a fresh drop of the same solution. Finally, the grid was washed by touching to a drop of water followed by immersion in two 10-ml beakers of water and one beaker of ethyl alcohol. After the sample was dried, it was rotary-shadowed with platinum. This protocol is designed for visualization of complete reaction mixtures, and no attempt was made to remove unreacted material. Although this approach should yield results that give a true insight into reaction components, it does lead to samples with a high background of unreacted proteins. Photography and measurement of filament and DNA length were performed as described previously (34).

ATPase Assay—A coupled spectrophotometric enzyme assay (35, 36) was used to measure the DNA-dependent ATPase activities of the wild-type RecA, RecAΔC6, RecAΔC13, RecAΔC17, and RecAΔC25 proteins. The regeneration of ATP from PEP and ADP was coupled to the oxidation of NADH and followed by the decrease in absorbance of NADH at 380 nm (380-nm wavelength was used so that the signal remained within the linear range of the spectrophotometer for the duration of the experiment). The assays were carried out on a Varian Cary 300 dual beam spectrophotometer equipped with a temperature controller and a 12-position cell changer. The cell path length and band pass were 0.5 cm and 2 nm, respectively. The NADH extinction coefficient at 380 nm of $1.21\ mM^{-1}\ cm^{-1}$ was used to calculate the rate of ATP hydrolysis.

The reactions were carried out at 37 °C in 25 mM Tris-OAc (80% cation), MES (33% anion), or Tris-OAc (56% cation) for a reaction pH of 7.3, 6.0, or 8.0, respectively, 1 mM DTT, 3 mM potassium glutamate, 10 mM $Mg(OAc)_2$, 5% (w/v) glycerol, an ATP regeneration system (10 units/ml pyruvate kinase, 2.2 mM PEP for reactions with single-stranded DNA or 3.0 mM PEP for reactions with duplex DNA), a coupling system (3 mM NADH and 10 units/ml lactate dehydrogenase), and the concentration of DNA and wild-type RecA, RecAΔC6, RecAΔC13, RecAΔC17, or RecAΔC25 proteins indicated in the table or figure legends. Unless otherwise noted, ATPase assays that included RecAΔC25 protein also included an additional 40 mM potassium glutamate. The

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aforementioned components were incubated for 10 min. The assay was initiated by the addition of the SSB protein (to 0.8 μM) and the concentration of ATP indicated in the figure legends. SSB protein was omitted from reactions with nicked circular dsDNA.

DNA Three-strand Exchange Reactions Promoted by the Wild-type and Deletion Mutant Proteins—Three-strand exchange reactions were carried out in 25 mM buffer (varied as indicated to alter pH), 1 mM DTT, 5% (w/v) glycerol, 3 mM potassium glutamate, and 10 mM $Mg(OAc)_2$. The buffers used were MES (33% anion, pH 6.0), MES (44% anion, pH 6.2), MES (55% anion, pH 6.4), MES (61% anion, pH 6.5), MES (76% anion, pH 6.9), HEPES (24% cation, pH 7.0), Tris-OAc (80% cation, pH 7.3), HEPES (39% cation, pH 7.3), HEPES (56% cation, pH 7.6), Tris-OAc (56% cation, pH 8.0), Tris-OAc (39% cation, pH 8.3), TAPS (44% anion, pH 8.4), CHES (17% anion, pH 8.5), Tris-OAc (20% cation, pH 8.7), TAPS (72% anion, pH 8.8), CHES (33% anion, pH 8.9) for the indicated reaction pH. In each case, the reported pH is the expected final pH of the reaction mixture, obtained by determining the pH of a model reaction mixture containing all reaction components but substituting TE buffer for the DNA additions and the appropriate protein storage buffers for each protein addition. Reactions also contained an ATP regeneration system of 10 units/ml pyruvate kinase and 3.2 mM PEP. All incubations were carried out at 37 °C. The following are final concentrations. The wild-type RecA, RecAΔC6, RecAΔC13, RecAΔC17, or RecAΔC25 protein (6.7 μM) was preincubated with 20 μM ϕX174 circular ssDNA for 10 min. SSB protein (2 μM) and ATP (3 mM) were then added, followed by another 10-min incubation. The reactions were initiated by the addition of ϕX174 linear dsDNA to 20 μM. A 10-μl aliquot was removed to use for a zero time point, The reaction was incubated, and at the indicated time points, 10-μl aliquots were removed and the reaction was stopped by the addition of 5 μl of a solution containing 60 mM EDTA, 6% SDS, 25% (w/v) glycerol, and 0.2% bromphenol blue. Samples were subjected to electrophoresis in 0.8% agarose gels with 1× TAE buffer, stained with ethidium bromide, and exposed to ultraviolet light. Gel images were captured with a digital CCD camera utilizing GelExpert software (Nucleotech). When indicated, the intensity of DNA bands was quantitated with the software package TotalLab version 1.10 from Phoretix.

RESULTS

Experimental Design—A set of C-terminal deletion proteins was constructed to systematically test the role of groups of acidic residues in DNA binding and DNA strand exchange reactions (Fig. 1). The RecAΔC6 deletion removes 6 amino acid residues from the C terminus, 3 of which are negatively charged: Glu^{347}, Glu^{350}, and Asp^{351}. The RecAΔC13 deletion removes an additional 7 residues, 3 of which contribute negative charges: Glu^{343}, Asp^{341}, and Asp^{340}. The RecAΔC17 deletion removes an additional 4 residues, 1 of which is negatively charged: Asp^{336}. The RecAΔC25 deletion removes an additional 8 residues but does not remove any further negative charges. Because the RecAΔC25 mutant (RecA5327) was previously characterized by Ogawa and co-workers (13), this construct provided a good basis with which to compare our results with published work. The RecAΔC25 protein exhibited a tendency to precipitate with loss of activity under standard reaction conditions. To stabilize the protein, 40 mM potassium glutamate was added to reactions with this mutant except where noted.

E. coli Strains Expressing RecA C-terminal Deletion Mutants Are Not UV- or Ionizing Radiation-sensitive but Are Mitomycin C-sensitive—We tested whether strains containing the C-terminal deletion mutants on the chromosome were more sensitive to DNA damage than a strain containing the full-length RecA protein. Ogawa and colleagues (37) previously determined that a strain expressing RecAΔC25 protein from a plasmid is not more UV-sensitive than a strain expressing the wild-type RecA protein. Fig. 3*A* shows that strains expressing the RecAΔC6, RecAΔC13, and RecAΔC17 proteins from the chromosome are also as resistant to UV irradiation as the strain expressing the wild-type protein.

We also wished to determine whether a more significant challenge to cellular DNA metabolism might reveal an *in vivo* defect in the strains harboring the truncated RecA proteins. We

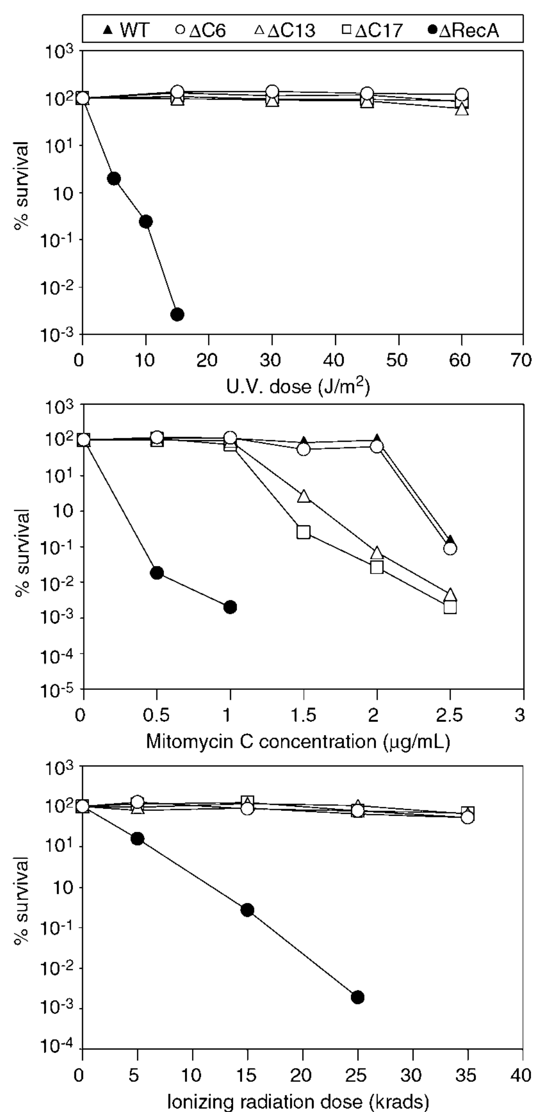

FIG. 3. **Survival curves for *E. coli* strains harboring the wild-type *recA* gene (*closed triangles*), no *recA* gene (*closed circles*), the *recA*ΔC6 gene (*open circles*), the *recA*ΔC13 gene (*open triangles*), or the *recA*ΔC17 gene (*open squares*) as a function of UV dose (*top*), mitomycin C concentration (*middle*), or ionizing radiation dose (*bottom*).** See "Experimental Procedures" for conditions. The Δ*recA* strain was extremely sensitive to these agents; therefore, cell survival could not be monitored above 15 J/m² UV, 1.0 μg/ml mitomycin C, or 25 krads of ionizing radiation.

thus examined the cells' sensitivity to the cross-linking agent mitomycin C and to ionizing radiation. The strain expressing RecAΔC6 was as resistant to mitomycin C as the strain expressing wild-type RecA protein. However, strains expressing the RecAΔC13 or RecAΔC17 proteins from the chromosome exhibited an enhanced sensitivity to this reagent. The strains harboring the RecAΔC13 or RecAΔC17 protein are ~1000-fold more sensitive to 2 μg/ml mitomycin C than the strain expressing wild-type RecA protein (Fig. 3*B*). This experiment was repeated twice with consistent results. It was not possible to test the RecAΔC25 mutant in this experiment, since it proved refractory to cloning on the chromosome using the protocol used for the other mutants (see "Experimental Procedures"). In contrast to the results obtained with mitomycin C, the presence of the C-terminal deletions had no evident effect on the sensitivity of cells to ionizing radiation (Fig. 3*C*).

The RecA C-terminal Deletion Mutant RecAΔC17 Forms Full and Extended Filaments on Circular ssDNA—In order to con-

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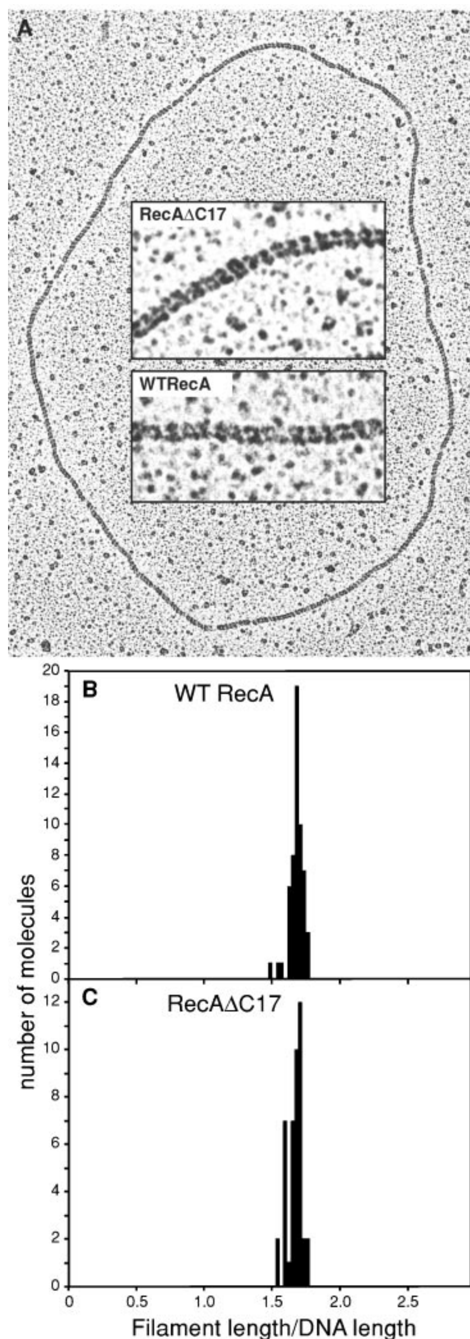

FIG. 4. **RecAΔC17-ssDNA nucleoprotein filaments analyzed by electron microscopy.** *A*, an electron micrograph showing the RecAΔC17 protein coating circular ssDNA to form a nucleoprotein filament. The *top inset* shows an enlargement of a portion of the RecAΔC17 full filament; the *bottom inset* shows an enlargement of a wild-type RecA filament (full filament not shown) formed under the same conditions as the mutant (see "Experimental Procedures"). Measured lengths of these filaments are shown as a histogram comparing the length of the wild-type RecA nucleoprotein filament (*B*) and the RecAΔC17 filament (*C*) with the length of duplex DNA (7.25 kilobase pairs).

firm that the RecA deletion mutants were forming complete, extended filaments, we analyzed RecAΔC17-ssDNA nucleoprotein filaments by electron microscopy and compared them with wild-type RecA-ssDNA filaments (Fig. 4*A*). Measurements indicate that both the RecAΔC17 and the wild-type RecA filaments are well extended by 1.66- and 1.67-fold, respectively, relative to double-stranded M13mp8 DNA (Fig. 4, *B* and *C*).

The ssDNA-dependent ATPase Activity of the RecA C-terminal Deletion Mutants—ATP hydrolysis by the RecA protein has been used in many studies as an indirect measure of DNA binding. This activity is almost entirely DNA-dependent under most reaction conditions and generally correlates well with other measures of DNA binding (16). The measured k_{cat} for ATP hydrolysis by RecA protein bound to ssDNA is ~30 min^{-1} and is reduced to under 20 min^{-1} when the RecA is bound to dsDNA or is promoting DNA strand exchange (3, 38, 39).

We measured the rates of ssDNA-dependent ATP hydrolysis using M13mp8 circular ssDNA cofactor for each C-terminal deletion mutant at pH 7.3, 6.0, and 8.0 (Table I). The rate of ATP hydrolysis was first examined as a function of wild-type and RecAΔC17 protein concentration in Fig. 5. The shape of the titration curves is similar for both proteins, although the somewhat lower rate of ATP hydrolysis seen at each point for the C-terminal mutant might be interpreted as reflecting a somewhat reduced intrinsic affinity for ssDNA. This type of data was obtained for all of the proteins, verifying that the concentration of each protein needed to measure the maximal rate of circular ssDNA-dependent ATP hydrolysis is approximately the same (data not shown). The apparent turnover number (k_{cat}, the number of ATP molecules hydrolyzed per RecA molecule per unit of time) given for each of the proteins in Table I was determined using V_{max} (μM ATP/min) rates at saturating RecA protein concentrations and dividing by the concentration of RecA binding sites in the DNA (assuming that all are bound by RecA). All of these RecA variants appear to have similar intrinsic rates of ssDNA-dependent ATP hydrolysis under these conditions.

The C Terminus Affects the Lag in dsDNA Binding Exhibited by the Wild-type RecA Protein—The wild-type RecA protein exhibits a long lag in binding to dsDNA, reflecting a slow nucleation step, at pH values close to and above physiological pH (15, 16). In order to determine which group or groups of negative charges in the C terminus contribute to this effect, we measured and compared the nicked circular dsDNA-dependent ATP hydrolysis rates of the wild-type RecA, RecAΔC6, RecAΔC13, RecAΔC17, and RecAΔC25 proteins (Fig. 6). The reactions were carried out at pH 7.3 (Fig. 6*A*) or at pH 6.0 (Fig. 6*B*). Potassium glutamate was not included in the RecAΔC25 reaction at pH 7.3, since the instability of the protein was less evident at this pH, and in this one case the rate of the reaction was greater in the absence of the added salt. The rates given in Fig. 6*C* were calculated using linear regression on the amount of ATP hydrolyzed during the steady-state achieved after binding appeared to be complete. The apparent k_{cat} was determined by assuming that all DNA binding sites on the DNA were occupied by RecA. The binding to dsDNA by the wild-type and RecAΔC6 was so slow as to make it unlikely that a saturated binding state had been achieved during the time of the experiment, and those rates are not included in Table I.

As expected, the wild-type RecA protein exhibited a long lag in DNA binding at pH 7.3, ≥170 min. The RecAΔC6 exhibited a long lag as well, ≥100 min. The major increase in the rate of binding was observed when 13 amino acid residues were deleted from the C terminus. The RecAΔC13 and RecAΔC17 mutants exhibited binding lags of about 24 and 20 min, respectively (Fig. 6*A*, *inset*). The RecAΔC25 protein bound faster than any of the others, exhibiting a lag of only about 7 min.

The lag exhibited in dsDNA binding by the wild-type RecA protein can be alleviated by lowering the pH of the reaction (15, 16). We repeated the dsDNA-dependent ATPase experiments at pH 6.05. As expected, the wild-type RecA protein bound rapidly to dsDNA at this lower pH (Fig. 6*B*). The lag in dsDNA binding was also reduced for all of the other proteins tested.

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TABLE I
ATP hydrolysis turnover numbers (k_{cat}) for the wild-type and C-terminal deletion mutant proteins

The reactions were carried out as described under "Experimental Procedures" and included 6 μM wild-type or mutant RecA protein and 8 μM M13mp8 circular ssDNA or 2 μM wild-type or mutant RecA protein and 4.2 μM nicked circular dsDNA. The apparent k_{cat} values listed in the table that include S.D. values were generated by averaging steady state rates from more than three experimental trials. The values with no S.D. were repeated twice with no more than 2 min^{-1} deviation. The values reported are the average of the two measurements. ND, not determined.

	$k_{cat(app)}$				
	cssDNA			dsDNA	
Protein	pH 7.3	pH 6.0	pH 8.0	pH 7.3	pH 6.0
		min^{-1}		min^{-1}	
WT RecA	30.3 ± 3.7	28.2	24.5	ND	22.0 ± 1.5
RecAΔC6	29.7 ± 2.7	29.3	21.8	ND	24.5 ± 1.9
RecAΔC13	30.1 ± 4.9	28.3	18.8	13.1 ± 2.4	22.0 ± 1.9
RecAΔC17	29.9 ± 4.5	29.9	22.2	14.9 ± 2.2	18.9 ± 0.5
RecAΔC25	28.9 ± 0.2	ND	ND	16.9 ± 1.7	18.3 ± 1.3

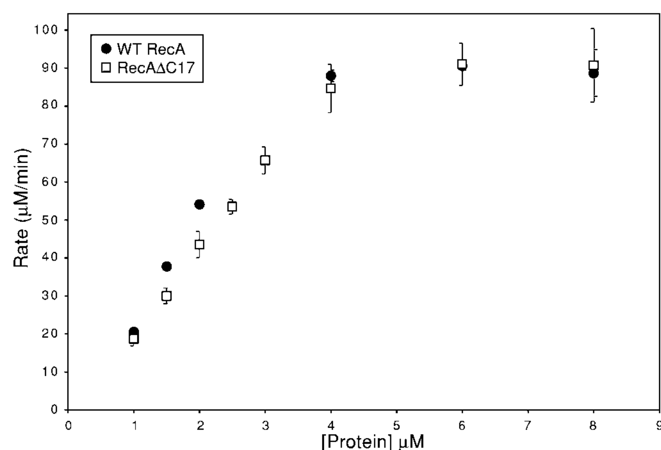

FIG. 5. **The rate of ATP hydrolyzed as a function of wild-type RecA (*circles*) or RecAΔC17 (*squares*) protein concentration.** These data are representative of the protein titration data collected for all mutants investigated in the current study. The reactions were carried out as described under "Experimental Procedures" at pH 7.3 and included 8 μM circular ssDNA, 3 mM ATP, 0.8 μM SSB protein, and the indicated concentrations of the wild-type and RecAΔC17 proteins. Each point on the titration curve is an average of three (wild type) and seven (ΔC17) independently collected data points. *Error bars* for the wild type and RecAΔC17 averaged data point are shown on the *right* and *left*, respectively.

The RecA C-terminal Deletion Mutants Promote DNA Strand Exchange of Bacteriophage φX174 Substrates—We determined the effect of the C-terminal deletion mutations on the fundamental RecA protein-promoted DNA strand exchange reaction (Fig. 7). The mutants are able to promote homologous pairing, indicated by the formation of joint molecules as evident in the agarose gels. Furthermore, the mutants were able to promote a complete DNA strand exchange reaction, as indicated by the production of nicked circular product. Under these conditions (pH 7.3), the effect that these C-terminal deletions appear to have on the RecA-facilitated DNA strand exchange reaction is in the kinetics and the extent of final product formation. In the wild-type RecA protein reaction, final nicked circular products appear in the 15-min time point. Significant amounts of these products first appear in the 30-min time point for RecAΔC6, RecAΔC13, and RecAΔC17 reactions and in the 60-min time point for the RecAΔC25 reactions. Furthermore, reactions promoted by the RecAΔC17 and RecAΔC25 proteins do not reach the same levels of final nicked circular product as the other proteins in 90 min, and the RecAΔC25 was consistently quite weak.

C-terminal Deletions of RecA Protein Dramatically Alter the pH-Reaction Profiles of RecA Protein-mediated DNA Strand Exchange Reactions—We repeated DNA strand exchange reactions at pH 6.0 (Fig. 8). The DNA strand exchange facilitated by the RecAΔC6 proteins was increased, somewhat stronger than the wild-type RecA reaction at this pH. Conversely, the RecAΔC13, RecAΔC17, and RecAΔC25 proteins were not able to promote a complete DNA strand exchange (evidenced by the lack of formation of final nicked circular product) at this lower pH, and even the formation of joint molecule intermediates was reduced.

The result shown in Fig. 8 triggered a broader investigation of the effects of the C-terminal deletions on the pH-reaction profile of DNA strand exchange. In an attempt to reduce the level of complex aggregates produced by RecAΔC13 and RecAΔC17, the ATP concentration was reduced to 400 μM in the experiment shown in Fig. 9. RecAΔC25 was not included in this analysis. The *top four panels* of Fig. 9 show the nicked circular product formation promoted in 90 min by the wild-type and variant proteins as a function of reaction pH. The final product yield is quantified for all four proteins in the *bottom panel* of Fig. 9. The wild-type protein exhibits a reaction optimum near pH 7, trailing off gradually at higher pH values until the reaction is eliminated at pH values above 8.6. The reactions seen with RecAΔC6 are similar although reproducibly stronger than wild-type reactions at low pH values. The entire pH-reaction profile is markedly shifted to higher pH values for the RecAΔC13 and RecAΔC17 mutant proteins (Fig. 9). Similar results were obtained in a set of trials at 3 mM ATP, although the RecAΔC6-promoted reaction did not fall off quite as abruptly at pH values above 7.5 (data not shown).

The pH-reaction profile for the wild-type protein exhibits more up and down fluctuations around the *smooth curve* drawn in the *bottom panel* of Fig. 9 than is evident in the curves for the C-terminal deletion mutant proteins. Significant scatter is often seen in results obtained in DNA strand exchange experiments. However, much of the variation seen here for the wild-type protein is not attributable to experimental scatter but instead represents reproducible variation (experiments were repeated four times, with S.D. not exceeding 6%), reflecting the use of different buffers in adjacent points (*e.g.* HEPES *versus* Tris). The fluctuations are most evident between pH 7 and 8.5. At a given pH, the particular buffer employed in the experiment can have a significant effect on the yield of strand exchange products, if all other reactants and parameters are held constant. However, notably, this effect is evident only with the wild-type protein. The deletion mutants exhibit no significant difference in strand exchange product yield with the different buffers of similar pH used here.

DISCUSSION

This study was carried out to more precisely define the effects of RecA protein C-terminal deletions on fundamental RecA protein functions both *in vivo* and *in vitro*. The C-terminal deletions of the *E. coli* RecA protein have much more

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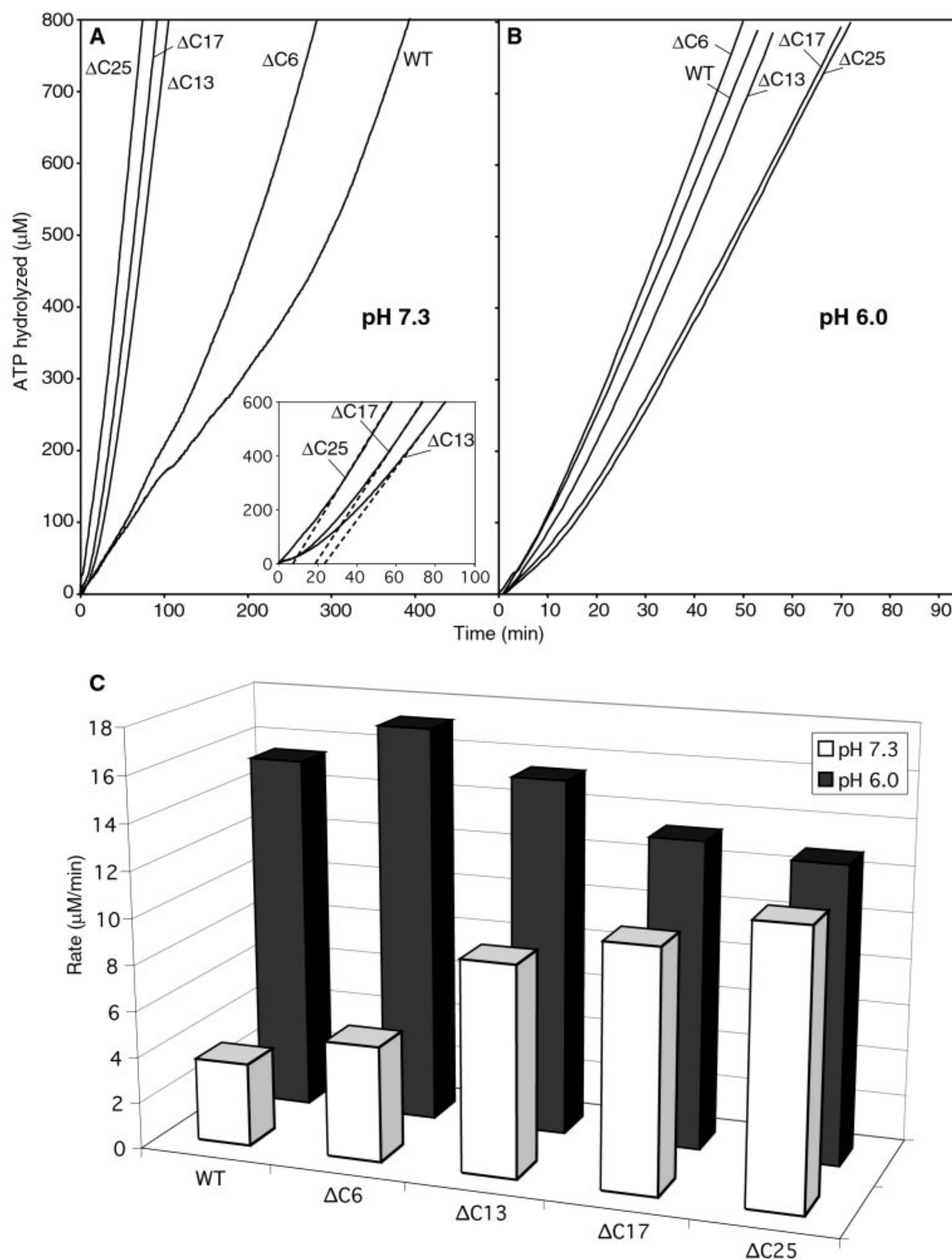

FIG. 6. **The effect of C-terminal deletions on the binding to dsDNA at pH 7.3 and 6.0.** The DNA cofactor is nicked circular duplex M13mp8 DNA. The reactions were performed under conditions described under "Experimental Procedures" at pH 7.3 (*A*) or pH 6.0 (*B*) and included 2 μM wild-type or mutant RecA protein, 4.2 μM nicked circular dsDNA, and 3 mM ATP. The *inset* to *A* is an expansion of the early times in the assay and is intended to highlight the difference in lag times between the RecAΔC25, RecAΔC17, and RecAΔC13 proteins. The *dotted lines* are extrapolations back to the *x* axis from the linear segments (reflecting the steady state) of the curved *lines* achieved at late reaction times. The maximum rates of ATP hydrolysis at pH 7.3 and 6.0 are plotted in the histogram shown in *C*. The rates indicated are averaged rates recorded over six (pH 7.3) or three (pH 6.0) independent experimental runs (one representative experiment for each pH is shown in *A* and *B*).

significant and complex effects on the activity of the protein than previously appreciated, signaling a more robust role of the C-terminal domain in RecA function. There are three major conclusions. First, we have identified the first *in vivo* deficiency of C-terminal RecA deletion mutants. Whereas strains harboring C-terminal deletion mutants of RecA are not more UV-sensitive than normal, as previously reported (17, 37), and are not more sensitive to ionizing radiation, they do exhibit a significant increase in sensitivity to the DNA cross-linker mitomycin C. Second, we can attribute much of the *in vitro* enhancement of binding to dsDNA, previously observed in RecAΔC25 (13), to deletion of residues between positions 339 (RecAΔC13) and 346 (RecAΔC6). A smaller but significant enhancement of binding is also observed when the residues between positions 327 (RecAΔC25) and 335 (RecAΔC17) are deleted. Third and finally, short C-terminal deletions have dramatic effects on the pH-reaction profile of the DNA strand exchange reaction. This result indicates that the C terminus of the wild-type protein affects the pK_a of at least two groups in other parts of the protein that are important in the DNA strand exchange reaction and suggests a potentially extensive and complex interaction between the C terminus and the rest of the protein.

The difference in the *in vivo* sensitivities to DNA damage caused by UV or ionizing irradiation and mitomycin C observed for the RecA C-terminal deletion mutants suggests that the C

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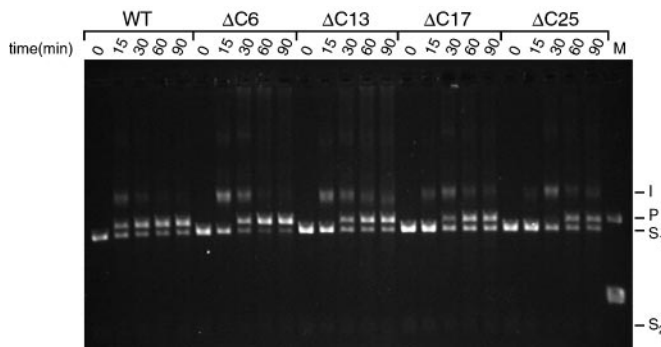

FIG. 7. **DNA strand exchange reactions promoted by the wild-type, ΔC6, ΔC13, ΔC17, and ΔC25 RecA proteins.** Reactions were carried out as described under "Experimental Procedures" at pH 7.3 and included 3 mM ATP. Aliquots of the reaction were removed and stopped at the times indicated *above* the individual *lanes* and subjected to agarose gel electrophoresis. The joint molecule intermediates (*I*) and nicked circular products (*P*) are distinguishable from the circular ssDNA (S_2) and the linear duplex DNA (S_1) substrates of the reaction by their relative mobilities. *Lane M*, nicked circular and supercoiled dsDNA marker.

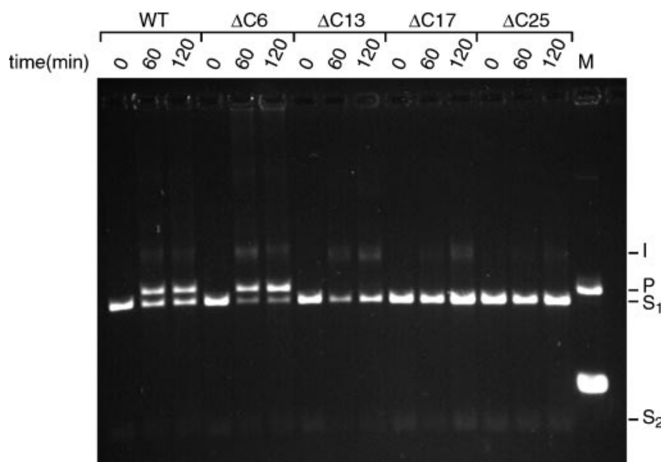

FIG. 8. **DNA strand exchange reactions promoted by the wild-type and mutant RecA proteins at pH 6.0.** The DNA strand exchange conditions are described under "Experimental Procedures" at pH 6.0 and included 3 mM ATP. The labels are detailed in the legend to Fig. 7. *Lane M*, nicked circular (*P*) and supercoiled dsDNA marker.

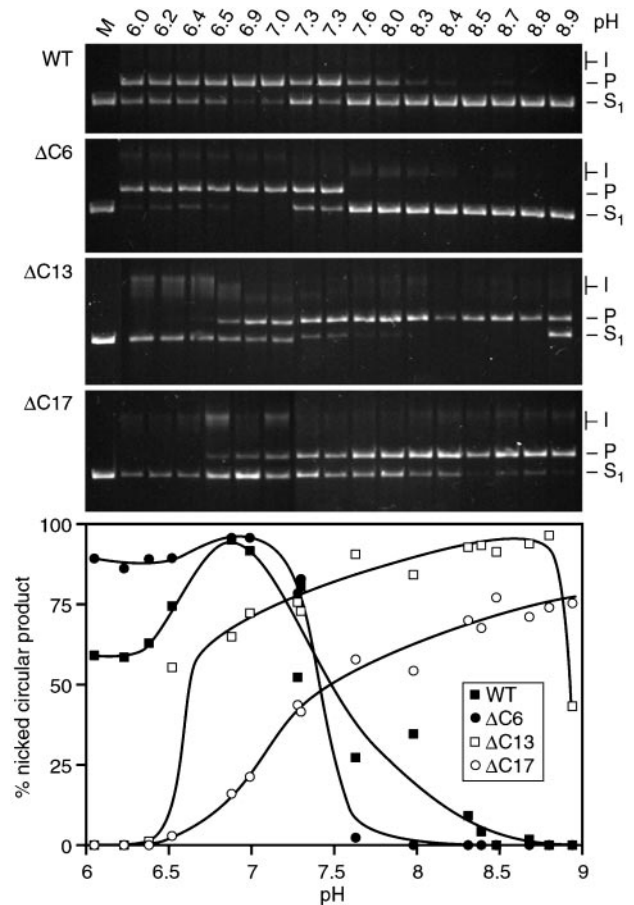

FIG. 9. **Dependence of the wild type- and mutant RecA protein-promoted DNA strand exchange reaction on pH.** DNA strand exchange reactions were performed under the conditions described under "Experimental Procedures" at the pH indicated and included 400 μM ATP. The *labels* are detailed in the legend to Fig. 7. *Lane M*, substrate linear dsDNA (S_1). In the *bottom panel*, the product formation by the wild type and the C-terminal deletion mutants, ΔC6, ΔC13, and ΔC17 is quantified as a function of pH.

terminus of RecA protein is critical to RecA function only when the cell faces particular DNA metabolic stresses. The C-terminal deletion mutants do not confer greater sensitivity to UV or ionizing radiation challenges. However, strains expressing the RecAΔC13 and RecAΔC17 proteins are 1000-fold more sensitive to 2 μg/ml of mitomycin C. The DNA-damaging agent mitomycin C is known to introduce interstrand cross-links into duplex DNA (40). The sensitivity observed in strains expressing the RecAΔC13 and RecAΔC17 mutant proteins is not evident until a threshold of 1 μg/ml of mitomycin C in the growth medium is reached. This suggests that the mutants have a deleterious effect on repair and cell survival only when the load of DNA cross-links exceeds a particular level. The repair of such cross-links is thought to require the recombinase function of RecA, the excinuclease function of the UvrABC complex and the strand synthesis function of the DNA polymerase I protein (41–43). It is possible that the direct action of RecA protein at the site of interstrand cross-link repair involves the C terminus of the protein, perhaps as an interaction site for other proteins involved in the repair process. The result reinforces the overall hypothesis that some RecA functions are quite specialized and needed only in certain circumstances (44).

This study was designed not only to examine the role of the C terminus of the RecA protein but also to determine more precisely which group or groups of negative charges located at the C terminus of the RecA protein are responsible for activity changes. The most significant change in activity previously reported for C-terminal deletion mutants is the improvement in the rate of nucleation onto dsDNA at physiological pH (13, 14). The major improvement in binding to dsDNA occurs in the transition from RecAΔC6 to RecAΔC13, which involves deletion of the negatively charged residues Glu^{343}, Asp^{341}, and Asp^{340}. However, a smaller but significant improvement in binding is seen when the RecA deletion is increased from 17 to 25 amino acid residues (residues 328–335), and there are no additional negatively charges removed from the polypeptide in this region. Thus, the slow binding to dsDNA by wild-type RecA protein may be ascribed in some measure to the removal of a protein segment that imparts some other property besides negative charge.

Some of the most dramatic effects of the C-terminal deletions are seen in the pH-reaction profile for DNA strand exchange. The wild-type RecA protein promotes a fairly facile strand exchange reaction between pH 6 and 7.5, with a maximum near pH 7. The yield of strand exchange products then declines gradually until reaction is largely eliminated above pH 8.6. Progressive deletion of 13 or 17 amino acid residues from the C terminus leads to a marked upward shift of the pH-reaction profile (RecAΔC13 and RecAΔC17) such that the strand exchange reaction is eliminated

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at low pH and enhanced at high pH.

The C-terminal deletions and the groups affected by them could be altering any of several steps of DNA strand exchange. The lack of strand exchange at high (wild-type and RecAΔC6) or low (RecAΔC13 and RecAΔC17) pH could reflect a disruption of RecA filament formation. However, over much of the pH range investigated, DNA pairing intermediates suggesting the presence of minimally functional filaments were present even when complete product formation was not observed. Alternatively, the C-terminal deletions might affect a conformational change to a form active in strand exchange, or ATP hydrolysis during DNA strand exchange. When RecA protein is bound to ssDNA, it is in a conformation that exhibits limited cooperativity and little exchange of RecA monomers between free and bound forms. When homologous duplex DNA is introduced, the conformation changes to a form that is more cooperative and more dynamic (38, 45), and the rate of ATP hydrolysis drops about one-third (39). ATP hydrolysis also becomes coupled to DNA strand exchange in this mode (32, 46–50). The C-terminal deletions could be affecting the ionization properties of groups critical to the conformation change, and/or to the hydrolysis of ATP in the new conformation.

The patterns evident in Fig. 9 are complex. However, the results suggest that the C terminus interacts with the rest of the protein and affects conformational transitions that must occur during RecA protein-mediated DNA strand exchange. We propose that there are multiple salt bridges that exist at least part of the time between Glu and Asp residues in the C terminus and positively charged groups elsewhere in the protein. The pK_a values of additional groups involved in DNA strand exchange may be affected by the changes in conformation that occur or do not occur as C-terminal residues are deleted. Multiple scenarios are possible, but a minimal working hypothesis might involve the ionization state of two different residues (both outside the C terminus), such that both the ascending and descending legs of the pH-reaction profile are shifted upward by multiple pH units. It is possible, of course, that the observed pH-reaction effects reflect the ionization of more than two groups. Recent work from the Record laboratory (51) has documented the importance of salt bridges in mediating the extensive binding of DNA to a protein surface. A similar phenomenon may mediate the binding of the duplex DNA to the RecA nucleoprotein filament during DNA strand exchange.

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