Biochemical Characterization of a Mutant RecA Protein Altered in DNA-Binding Loop 1[†]

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ABSTRACT: The double substitution of Glu156 with Leu and Gly157 with Val in the *Escherichia coli* RecA protein results in a severely reduced level of recombination and constitutive coprotease behavior. Here we present our examination of the biochemical properties of this mutant protein, RecA N99, in an effort to understand its phenotype and the role of loop 1 (L1) in RecA function. We find that RecA N99 protein has reduced single-stranded DNA (ssDNA)-dependent ATP hydrolysis activity, which is not as sensitive to the presence of SSB protein as wild-type RecA protein. RecA N99 protein is also nearly unable to utilize duplex DNA as a polynucleotide cofactor for ATP hydrolysis, and it shows both a decreased rate of association with ssDNA and a diminished capacity to bind DNA in the secondary binding site. The mutant protein has a corresponding reduction in DNA strand exchange activity, which probably results in the decrease in recombination activity *in vivo*. The constitutive induction of the SOS response may be a consequence of the impaired ability to repair damaged DNA, resulting in unrepaired ssDNA which can act as a cofactor for the cleavage of LexA repressor. These findings point to an involvement of L1 in both the primary and secondary DNA binding sites of the RecA protein.

The RecA protein is a multifunctional enzyme that is important in homologous genetic recombination and the repair of DNA damage. RecA protein catalyzes the pairing and exchange of homologous DNA molecules and the autocleavage of the LexA repressor, which induces the expression of a set of unlinked genes (*1−4*), including *recA*, to begin the SOS response. The catalytic activities of RecA protein require the formation of a nucleoprotein filament consisting of RecA protein, ssDNA,[1] and ATP. To understand the molecular mechanisms of the various enzymatic activities of the RecA protein, it is important to identify the different regions within the protein structure that are responsible for these functions, particularly the DNA binding sites and the repressor binding site.

RecA protein has two DNA binding sites, a primary site responsible for filament formation and a secondary site for the nonspecific binding of dsDNA necessary in the search for homology (*5*). The secondary DNA binding site has been shown to have both a synaptic and postsynaptic role; it acts to bind the incoming dsDNA for the homology search and

also to stabilize the DNA strand exchange product by binding the displaced ssDNA (*6, 7*).

Examination of the RecA protein crystal structure permits speculation regarding the location of these DNA binding sites (*8*). Undefined in the crystal structure are two disordered loops, loop 1 (L1) and loop 2 (L2), which correspond to residues 157−164 and 195−209, respectively. These loops, which are located close to the helical axis of the polymer, were proposed to be involved in DNA binding (*8*). DNA has been proposed to reside in the interior of the filament, as visualized by electron microscopy (*9, 10*). L1 is on the inner face of the polymer, and L2 is on the upper lip of the groove in the filament.

The DNA binding sites were defined experimentally by cross-linking RecA protein to the bound DNA. Among the residues identified by Morimatsu and Horii, using conditions allowing for binding in only the primary site, were residues 257−280, which overlap with L2 (*11, 12*). A study by Rehrauer and Kowalczykowski, which used a protein:DNA stoichiometry allowing for binding in both the primary and secondary sites, did not identify residues within either L1 or L2 as interacting with the bound DNA, but instead identified residues spanning the major domain between the loops (*13*). Using excess protein, presumably allowing only primary site binding, Malkov and Camerini-Otero found residues within both L1 and L2 cross-linked to the DNA (*14*). Finally, varying concentrations of DNA were used by Wang and Adzuma to distinguish between the binding sites; they identified L1 as the primary DNA binding site and L2 as the secondary site (*15*). The findings within this collection of DNA cross-linking studies are not consistent with one

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[1] Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; SSB protein, ssDNA-binding protein; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate).

another regarding the assignment of the primary and secondary DNA binding sites.

The biochemical behavior of several RecA mutants provides some insight regarding assignments of the role of L1 and L2 in DNA binding. RecA430 protein contains a mutation in L2 and has altered ssDNA binding properties, which indicates a role in the primary DNA binding site (16–19). On the other hand, a different L2 mutant, RecA E207Q protein, is competent in biochemical activities associated with the primary DNA binding site; however, this protein lacks secondary DNA binding site activity (20), which points to L2 being part of the secondary DNA binding site.

Mutants with substitutions in L1, such as RecA1602 and RecA1203 proteins, display constitutive coprotease activity and lack recombination activity, indicating a functional primary site and defective secondary site (21). These mutants suggest an involvement of L1 in the secondary DNA binding site or as part of a conformational switch that is essential for exchange of DNA strands.

The binding site of the LexA repressor on the RecA protein filament was examined using electron microscopy (10). This study visualized a complex of a noncleavable LexA repressor analogue and a RecA protein filament formed on dsDNA, and showed that the LexA repressor is bound in the groove of the RecA nucleoprotein filament. Comparison of the electron microscopy structure to the crystal structure identified residues 156–165 and 229–243 as regions of interaction with the bound LexA repressor; the former set contains residues within L1 (8, 10). The interaction of the LexA repressor with residues in L1 is substantiated by the substitution of residues in this region which results in mutants with constitutive coprotease activity (21, 22). Substitution of Gly157 by Nastri and Knight (22) was shown to result in coprotease constitutive mutants with decreased recombination activity, fortifying the suggestion that L1 is the secondary DNA binding site with an overlapping function as the LexA repressor binding site. This functional overlap in the RecA protein structure is further substantiated by the observation that excess ssDNA can inhibit the rate of LexA repressor cleavage (23–25). Inhibition of cleavage activity is also seen with the addition of dsDNA to the reaction mixture (25). Additionally, the mutually exclusive nature of LexA repressor cleavage and the binding of a second DNA molecule to the RecA nucleoprotein filament was shown by the interference of an uncleavable LexA repressor analogue in the DNA strand exchange reaction *in vitro* (26).

To investigate the role of the L1 region in recombination and LexA repressor cleavage, a series of mutant RecA proteins with substitutions in residues 152–159, which are neighboring or within L1, was created by Nastri and Knight (22). The recombinational repair and LexA repressor coprotease activities of these mutants were assayed *in vivo*, and it was determined that Glu154 is very sensitive to substitution and that mutations at Gly157 and Glu158 are hot spots for constitutive coprotease activity.

A mutant protein of particular interest from this study is RecA N99 protein, a double mutant with Glu156 substituted with Leu and Gly157 substituted with Val (22). Nastri and Knight designated this mutant as being constitutive for coprotease activity and Rec$^{+/-}$ (no survival was detected in the presence of 0.3 μg/mL mitomycin C, but 8% survived upon exposure to UV light) (22). Here we describe the *in*

vitro characteristics of the RecA N99 protein, in comparison to those of wild-type RecA protein, to account for its phenotype. Presumably, an active filament is formed *in vivo*, as indicated by the presence of coprotease activity, which suggests that a defect in the secondary DNA binding site is responsible for the altered recombination activity. An examination of DNA binding and DNA strand exchange activity indicates that the impaired recombinational repair phenotype of RecA N99 protein results from its impaired ability to effectively bind a second DNA molecule to the nucleoprotein filament. This conclusion is in accord with the suggestion that L1 is part of the secondary DNA binding site (8). Additionally, measurements of ATP hydrolysis activity and the competition with SSB protein suggest an alteration in the primary DNA binding site. A possible result of the impaired recombinational repair activity of RecA N99 protein is the constitutive coprotease activity, which may be due to the chronic presence of unrepaired ssDNA in the cell.

EXPERIMENTAL PROCEDURES

Chemicals and Buffers. All chemicals were reagent grade, and buffers were prepared with Barnstead Nanopure water. BCA protein assay reagents were purchased from Pierce and used according to recommended standard reaction conditions. Stop buffer (10×) contains 40% Ficoll, 0.2 mM EDTA, 20% SDS, 0.5 mg/mL bromophenol blue, and 0.5 mg/mL xylene cyanol FF. Gel loading buffer (GLB, 10×) contains 20% Ficoll and 0.1% bromophenol blue.

DNA. Circular M13 mp7 ssDNA was prepared from bacteriophage M13mp7 as described previously (27). Duplex M13 mp7 DNA was purified by alkaline lysis followed by cesium chloride ultracentrifugation (28, 29). Purified DNA was subsequently linearized with *Eco*RI restriction endonuclease. Concentrations of ssDNA and dsDNA were determined by using extinction coefficients at 260 nm of 8784 and 6500 M^{-1} cm^{-1}, respectively. All DNA concentrations are expressed in nucleotides. Oligonucleotide 2 was provided by A. Mazin of this laboratory (6). Plasmid pTRecA322 was obtained from K. Knight (22).

Proteins. Wild-type RecA protein was purified from strain GE1710 following a protocol based on spermidine acetate precipitation (30). All purification steps were performed at 4 °C. Cells were suspended in 4 mL/g of a buffer containing 50 mM Tris-HCl (pH 7.5), 25% (w/v) sucrose, 25 mM NaCl, 5 mM EDTA, and 1 mM DTT and then lysed using a French press. Phenylmethanesulfonyl fluoride (PMSF) was added before and during lysis to a final concentration of 10 μL/ mL. The protein in the cleared lysate was precipitated by the addition of a 10% solution of polyethyleneimine in water to a final concentration of 0.5%. The resulting pellet was extracted several times with R buffer [20 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 0.1 mM dithiothreitol, and 0.1 mM EDTA] containing 150 mM ammonium sulfate and then with R buffer containing 300 mM ammonium sulfate. The protein in the supernatant from the latter set of extractions was precipitated by the addition of ammonium sulfate to a concentration of 0.3 g/mL. The protein pellet was dissolved in phosphate buffer [20 mM KH$_2$PO$_4$/K$_2$HPO$_4$ (pH 6.5), 10% glycerol, 0.1 mM DTT, and 0.1 mM EDTA] with 200 mM NaCl, dialyzed into the same buffer, and then passed over a Whatman P-11 phosphocellulose column. The pooled flow-through fractions were precipitated again by the addition of

ammonium sulfate, as before. The resulting pellet was resuspended in spermidine acetate buffer [20 mM spermidine acetate (pH 7.5), 7 mM spermidine (pH 7.5), and 0.1 mM DTT] and dialyzed extensively into several changes of the same buffer. The resulting precipitate was pelletted, then dissolved in phosphate buffer containing 25 mM EDTA, and dialyzed into the same buffer and then into phosphate buffer containing 50 mM NaCl. The solution was applied to a DNA−cellulose column equilibrated with phosphate buffer containing 50 mM NaCl, and the RecA protein was eluted with phosphate buffer containing 250 mM NaCl and 1 mM ATP. The protein was again precipitated by the addition of ammonium sulfate, then dissolved in Tris buffer [20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 0.1 mM DTT] containing 100 mM NaCl, and dialyzed into the same buffer. The protein solution was then applied to a Mono-Q column equilibrated in the same buffer and eluted with a NaCl gradient in Tris buffer. The protein-containing fractions were checked for nuclease activity, pooled, and then precipitated with the addition of ammonium sulfate. The final pellet was dissolved in R buffer containing 1 mM DTT and dialyzed into the same buffer. The concentration of the protein was determined using an extinction coefficient at 280 nm of 2.7 $\times 10^4$ M^{-1} cm^{-1}. This procedure was used to purify wild-type RecA protein, a variety of mutant RecA proteins (*18*, *31−33*), and a similar procedure was used to purify human Rad51 protein (*34*).

RecA N99 protein (*22*) was expressed from a pTRecA322 plasmid derivative transformed into SCK322, which contains a *recA*, *sbcB* background. All purification steps were performed at 4 °C. Cells were suspended in a buffer containing 50 mM Tris-HCl (pH 7.5), 25% (w/v) sucrose, 25 mM NaCl, 5 mM EDTA, and 1 mM DTT and then lysed using a French press. The protein in the cleared lysate was precipitated by the addition of a 10% solution of polyethyleneimine in water to a final concentration of 0.5%. The resulting pellet was extracted several times with R buffer [20 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 0.1 mM dithiothreitol, and 0.1 mM EDTA] containing 50 mM ammonium sulfate and then with R buffer containing 150 mM ammonium sulfate. The protein in the supernatant from the latter set of extractions was precipitated by the addition of ammonium sulfate to a concentration 0.3 g/mL. The protein pellet was dissolved in phosphate buffer [20 mM KH_2PO_4/K_2HPO_4 (pH 6.5), 10% glycerol, 0.1 mM DTT, and 0.1 mM EDTA] with 200 mM NaCl and passed over a Whatman P-11 phosphocellulose column. The pooled flow-through fractions were dialyzed against phosphate buffer with no EDTA or NaCl, then applied to a hydroxyapatite column (Bio-Rad), and eluted with a phosphate gradient at a concentration of approximately 140 mM. The pooled fractions were dialyzed against Tris buffer [20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 0.1 mM DTT] with 500 mM NaCl, then loaded onto a Cibacron Blue column, and eluted with a step gradient in NaCl concentration to 1 M. Following dialysis against Tris buffer containing 100 mM NaCl, the protein-containing fractions were applied to a Mono Q column (Amersham Pharmacia Biotech) and eluted with a sodium chloride gradient in Tris buffer at approximately 330 mM. After dialysis against Tris buffer containing 100 mM NaCl, the final step was application of the protein-containing fractions to a Heparin HiTrap column (Amersham Pharmacia

Biotech) equilibrated with Tris buffer containing 100 mM NaCl and elution with 500 mM NaCl in Tris buffer, which resulted in concentration of the protein and removal of the remaining nuclease contamination. The concentration of the protein was determined using the BCA protein concentration assay with wild-type RecA protein as a standard. The yield was approximately 0.5 mg per 70 g of cells, and the purity was determined to be >95% by SDS−PAGE followed by staining with Coomassie Brilliant Blue.

SSB protein was purified from strain RLM727 following the method described in ref *35*. An extinction coefficient at 280 nm of 3.0×10^4 M^{-1} cm^{-1} was used to determine protein concentration (*36*).

Lactate dehydrogenase and pyruvate kinase were purchased as ammonium sulfate suspensions from Sigma. For use in the assays, a sample of the suspension was centrifuged, the supernatant decanted, and the resulting protein pellet dissolved in reaction buffer.

ATPase Assay. A spectrophotometric assay that couples the production of ADP to the oxidation of NADH was used to monitor the hydrolysis of ATP (*37*, *38*). Reactions were carried out at 37 °C in a buffer containing 25 mM Tris acetate (pH 7.5), 0.1 mM dithiothreitol, 10 mM magnesium acetate, 1 mM ATP, 1.5 mM phosphoenolpyruvate, 0.2−0.3 mg/mL NADH, 30 units/mL pyruvate kinase, and 30 units/mL lactate dehydrogenase. The DNA (3 μM), either poly(dT) or M13 ssDNA, was incubated with the indicated concentrations of RecA protein. Experiments with M13 ssDNA employed the addition of 0.6 μM SSB protein after a steady state of hydrolysis had been reached with 3 μM DNA and 1.5 μM RecA protein, or the DNA was precoated with SSB protein for 5 min prior to the addition of RecA protein (an equivalent amount of active RecA N99 protein was used). The lag times were determined by extending the linear portion of the trace, after a steady state had been obtained, back to zero and establishing the point of intersection.

Reactions that included duplex DNA were carried out in a buffer containing 25 mM MES-NaOH (pH 6.2), 1 mM ATP, 10 mM magnesium acetate, and 0.1 mM DTT. To conserve protein and thereby allow comparison of all assays using a single preparation of mutant protein, thin-layer chromatography was used. Aliquots of a reaction mixture containing 1.5 μM linear M13 dsDNA and 0.75 μM RecA were analyzed using thin-layer chromatography on polyethyleneimine−cellulose sheets developed with 1 M formic acid and 0.5 M LiCl (*39*).

Gel Electrophoretic Assay for DNA Binding. The binding of oligonucleotide-length DNA by RecA protein was observed in a gel electrophoretic assay (*6*, *7*). Oligomeric ssDNA labeled with ^{32}P at the 5′-end, 63 nucleotides in length and of mixed base composition, at 3 μM, was incubated for 12 min at 37 °C with varying concentrations of RecA protein (the active concentration of RecA N99 protein is indicated) in a buffer containing 33 mM HEPES (pH 7.0), 1.2 mM magnesium acetate, 2 mM dithiothreitol, 1 mM ATPγS, and 100 μg/mL BSA. The magnesium ion concentration was then increased to 15 mM, and incubation was continued for a further 10 min. GLB was added to the reaction, and it was immediately loaded onto a 10% polyacrylamide gel. The gels were analyzed on a Molecular Dynamics Storm 840 PhosphoImager using Image-QuaNT software.

Etheno DNA Binding. The size of the binding site of RecA protein was determined by titration of etheno M13 DNA, monitoring the increase in fluorescence upon binding (*18*). In a buffer consisting of 25 mM Tris-HCl (pH 7.5), 0.1 mM dithiothreitol, 1 mM ATPγS, and 4 mM magnesium chloride, at 25 °C, the fluorescence of 3 μM etheno M13 DNA was measured upon addition of increasing amounts of RecA protein (active amounts of RecA N99 protein are shown).

The kinetics of association of RecA protein with etheno M13 DNA were measured similarly. Assays were started by addition of 0.1 μM RecA protein, or a comparable amount of active RecA N99 protein, to 3 μM etheno M13 DNA, and the time course of the fluorescence increase accompanying protein binding to the ssDNA was measured (*18*).

DNA Strand Exchange Assay. Reactions for examining DNA strand exchange activity followed a protocol designed by Menetski et al. (*40*). In these reactions, M13 ssDNA (1.67 μM) and SSB protein (0.15 μM) were incubated for 10 min at 37 °C in a buffer containing 25 mM Tris acetate (pH 7.5), 1 mM dithiothreitol, and 4 mM magnesium acetate. This was followed by the addition of 1 mM ATPγS and 1 μM RecA protein (or an equivalent amount of active RecA N99 protein). After a further 10 min incubation, the reaction was started with the addition of 5′-^{32}P-radiolabeled 1.67 μM linear M13 dsDNA, and aliquots were removed from the reaction at the indicated times (*40*). The reaction was stopped by addition of the aliquots to 10× stop buffer. Reactions were analyzed by electrophoresis through a 1% agarose gel. The gels were analyzed on a Molecular Dynamics Storm 840 PhosphoImager using Image-QuaNT software.

LexA Repressor Cleavage Assay. The extent of cleavage of the LexA repressor was measured in a buffer containing 25 mM Tris-HCl (pH 7.5), 0.1 mM dithiothreitol, 10 mM magnesium chloride, 1 mM ATPγS, 50 mM sodium chloride, and 12.5 units/mL pyruvate kinase. Poly(dT) (3 μM) was incubated with 1 μM RecA protein (or an equivalent concentration of active RecA N99 protein) for 2 min at 37 °C. The indicated amount of excess poly(dT) was then added as a competitor for binding, and 10 μM LexA repressor was added to start the reaction. Incubation was continued for 20 min at 37 °C and stopped by the addition of 10× stop buffer. Reaction products were analyzed by SDS−PAGE on a 15% polyacrylamide gel and analyzed using Gel Pro Analyzer software (Media Cybernetics, L.P.). The percentage of LexA repressor cleaved was calculated as the sum of intensities of the cleavage products divided by the sum of the intensities of the cleaved and uncleaved products or by disappearance of the LexA repressor band intensity, normalized to the amount of RecA protein in the lane.

RESULTS

RecA N99 Protein Has ssDNA-Dependent ATP Hydrolysis Activity, Indicating Nucleoprotein Filament Formation. The effect of RecA protein concentration on ATP hydrolysis activity is shown in Figure 1. The sizes of the DNA binding sites determined from these data are approximately 3:1 (nt: protein monomer ratio) for wild-type RecA protein and apparently 1:1 for RecA N99 protein. This assay measures the extent of binding of DNA to the primary site of RecA protein, since only binding to this site affects ATPase activity. This result indicates that this RecA N99 protein preparation
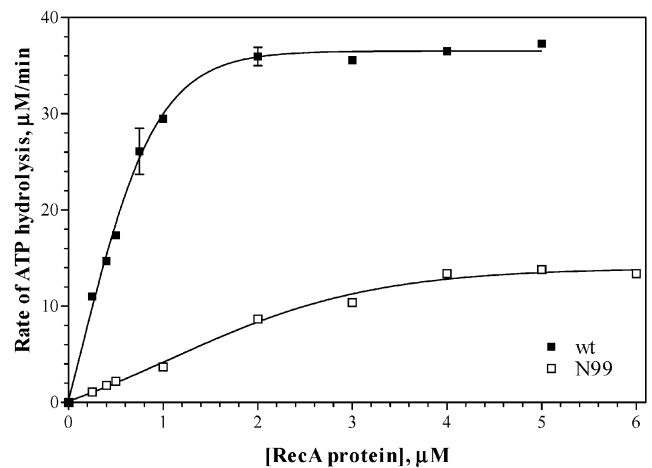

FIGURE 1: RecA N99 protein has reduced ssDNA-dependent ATP hydrolysis activity. Poly(dT) (3 μM) was incubated with varying concentrations of RecA protein, as noted, and the rates of ATP hydrolysis were measured. The actual (uncorrected for the presence of inactive protein) concentrations of RecA N99 protein are indicated.

either is one-third active or has a different apparent binding site size. To distinguish between these possibilities, subsequent RecA N99 protein preparations were examined, and these resulted in different site size measurements (data not shown); however, the maximum observed rate of hydrolysis was always constant. These results suggest that a variable fraction of protein is active within each preparation but that the limiting activity, k_{cat}, is constant. Because of an intrinsic instability that is unique to the RecA N99 protein, we were unable to isolate protein that was 100% active. For these reasons, we believe that the RecA N99 protein preparation shown is one-third active based on an expected binding site size of 3:1; additional data shown below support this interpretation.

The maximum rate of hydrolysis achieved by RecA N99 protein is significantly lower than that of wild-type RecA protein, approximately 14 and 36 μM/min, respectively. The observation that the maximum rate of ATP hydrolysis is the same for different preparations of RecA N99 protein (data not shown) suggests that the inactive portion of RecA N99 protein in each preparation does not interfere with the active portion. This inference is further substantiated in the following sections describing DNA binding, which show that a comparable amount of active RecA N99 protein is required to fully saturate the DNA and the inactive portion does not bind. This measure of active protein, i.e., based on the observed site size derived from ATP hydrolysis, relative to the expected site size, was used for all RecA N99 preparations, and only the active concentration is indicated in the subsequent sections and figures.

Duplex DNA Barely Stimulates the ATP Hydrolysis Activity of RecA N99 Protein. The capacity of RecA N99 protein to use duplex DNA as a polynucleotide cofactor for ATP hydrolysis activity was examined (Figure 2). In contrast to wild-type RecA protein, which is able to bind duplex DNA and hydrolyze ATP at an apparent rate of 3 μM/min (*41−44*), RecA N99 protein, at the same concentration of active protein, shows almost no hydrolysis activity. The observed rate is 0.4 μM/min (the rate of hydrolysis in the absence of DNA is <0.1 μM/min), which indicates a minimal amount
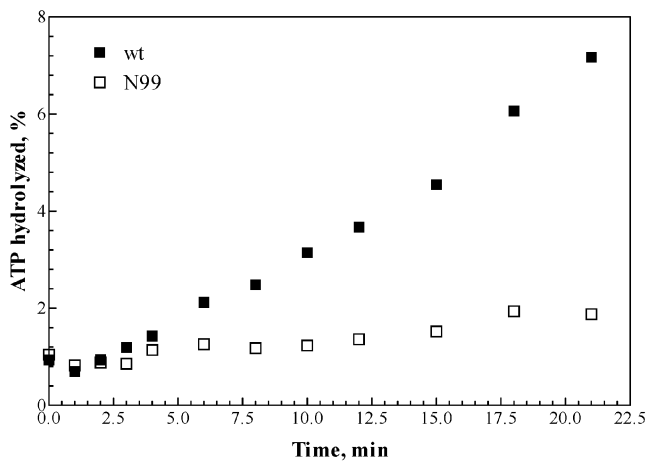
FIGURE 2: RecA N99 protein has severely impaired dsDNA-dependent ATP hydrolysis activity. Aliquots of a reaction mixture containing 1.5 μM linear M13 dsDNA and 0.75 μM RecA (the active concentration, in the case of RecA N99 protein) were taken at indicated times and analyzed using thin-layer chromatography on polyethyleneimine−cellulose sheets.

of RecA N99 binding to the duplex DNA under these conditions.

Gel Electrophoresis Reveals that RecA N99 Protein Has a Defective Secondary DNA Binding Site. A gel electrophoresis assay was used to measure the extent of binding of RecA protein to DNA oligonucleotides by monitoring the change in electrophoretic mobility of the bound DNA, as illustrated in Figure 3. This assay measures the level of binding of ssDNA to both the primary and secondary DNA sites of the RecA protein (7). It was previously shown that wild-type RecA protein could bind 2 equiv of 3 nucleotides each of ssDNA. This meant that RecA protein would bind 7 ($\pm$1) nucleotides of ssDNA by this physical criterion, whereas ATPase activity assays revealed a binding stoichiometry of only 3 ($\pm$1) nucleotides (because ATPase activity is activated only by binding to the primary site, and not to the secondary site). The stoichiometries obtained from the gel assay in Figure 3 are 8:1 and 4:1 (nucleotide:protein monomer) for wild-type RecA protein and RecA N99 protein, respectively. As stated above, the observed binding stoichiometry for wild-type RecA protein is expected to be approximately 2-fold greater than that obtained from the ATPase assay (Figure 1), because of binding of an added equivalent of DNA (approximately 3 nucleotides) to the secondary site of the RecA protein. On the other hand, the binding stoichiometry for RecA N99 protein from this assay [4 ($\pm$1) nucleotides] is nearly the same as that measured in the ATPase assay [3 ($\pm$1) nucleotides] (having been corrected for inactive protein in both assays), suggesting that binding to the secondary DNA binding site of RecA N99 protein is too weak to be observed in this assay. This assay also confirms that the inactive portion of RecA N99 protein does not bind the DNA, consistent with the absence of interference by the inactive fraction in the ATP hydrolysis measurements and further validating the assumption regarding protein activity.

The Binding of RecA N99 Protein to Etheno M13 DNA Confirms the Defect in the Secondary DNA Binding Site. The increase in the relative fluorescence of etheno M13 DNA upon addition of RecA protein was also used to determine the binding stoichiometry (45). The stoichiometries measured

by this method are 8.3:1 and 4.8:1 (nucleotide:protein monomer) for wild-type RecA protein and RecA N99 protein, respectively (Figure 4). This value for wild-type RecA protein is within the range observed with this method and reflects binding of etheno DNA to both the primary and secondary DNA binding sites (20, 45, 46). The apparent stoichiometry determined for RecA N99 protein is approximately half of that for wild-type RecA protein (after correction for activity). This result confirms the idea that RecA N99 protein lacks secondary site binding (20). Again, the inactive portion of RecA N99 protein is not observed to bind the DNA; if this portion of protein had bound the DNA, saturation of the DNA would be observed at a lower protein concentration. The binding of RecA N99 protein and wild-type RecA protein resulted in a comparable increase in fluorescence, indicating either that the same extended DNA structure was formed by each protein or that the fluorescence increase from binding solely in the primary site is equal to the increase from binding in both the primary and secondary sites. Finally, the binding of etheno DNA by RecA N99 protein was also assessed in the absence of nucleotide cofactor; however, the change in fluorescence was very low. The final increase in relative fluorescence was <10%, suggesting that RecA N99 protein is unable to bind ssDNA under these conditions.

Effect of SSB Protein on ssDNA-Dependent ATPase Activity. SSB protein stimulates M13 ssDNA-dependent ATP hydrolysis by RecA protein because more ssDNA becomes available for binding upon the removal of secondary structure by SSB protein (38, 47, 48). However, SSB protein affects ATP hydrolysis by RecA N99 protein in a manner different from that of wild-type RecA protein (Table 1): wild-type RecA protein activity is stimulated by the addition of SSB protein, whereas RecA N99 protein is inhibited slightly.

The order of addition of RecA protein and SSB protein to ssDNA affects the observed activity of RecA protein. Prior addition of SSB protein inhibits the ATP hydrolysis activity of RecA protein, resulting in a lag in the appearance of this activity and a reduced rate of hydrolysis as shown in Figure 5 (38, 49). The final rates of ATP hydrolysis (although these are not the final steady-state values) are reduced for both wild-type RecA protein and RecA N99 protein (17 and 6 μM/min, respectively), compared to the rates obtained by addition of SSB protein following the addition of RecA protein (see Table 1); however, the relative decrease in the rate is much greater for wild-type RecA protein.

The Association of RecA N99 with ssDNA Is Slower than That of Wild-Type RecA Protein. The rate of association of RecA protein with etheno M13 DNA was examined because this ssDNA binding property reflects the ability of RecA protein to compete with SSB protein (18, 33). The change in the intrinsic fluorescence of etheno M13 DNA upon addition of a subsaturating amount of RecA protein is shown in Figure 6. The time for the fluorescence to reach its maximum, indicating that the RecA protein has bound the DNA, is longer for RecA N99 than for wild-type RecA protein. RecA N99 protein is approximately 6-fold slower in its association with ssDNA, demonstrating that the mutations in the L1 region affect the rate of association.

RecA N99 Protein Exhibits Diminished DNA Strand Exchange Activity. DNA strand exchange activity was measured in the presence of ATPγS, since no activity was
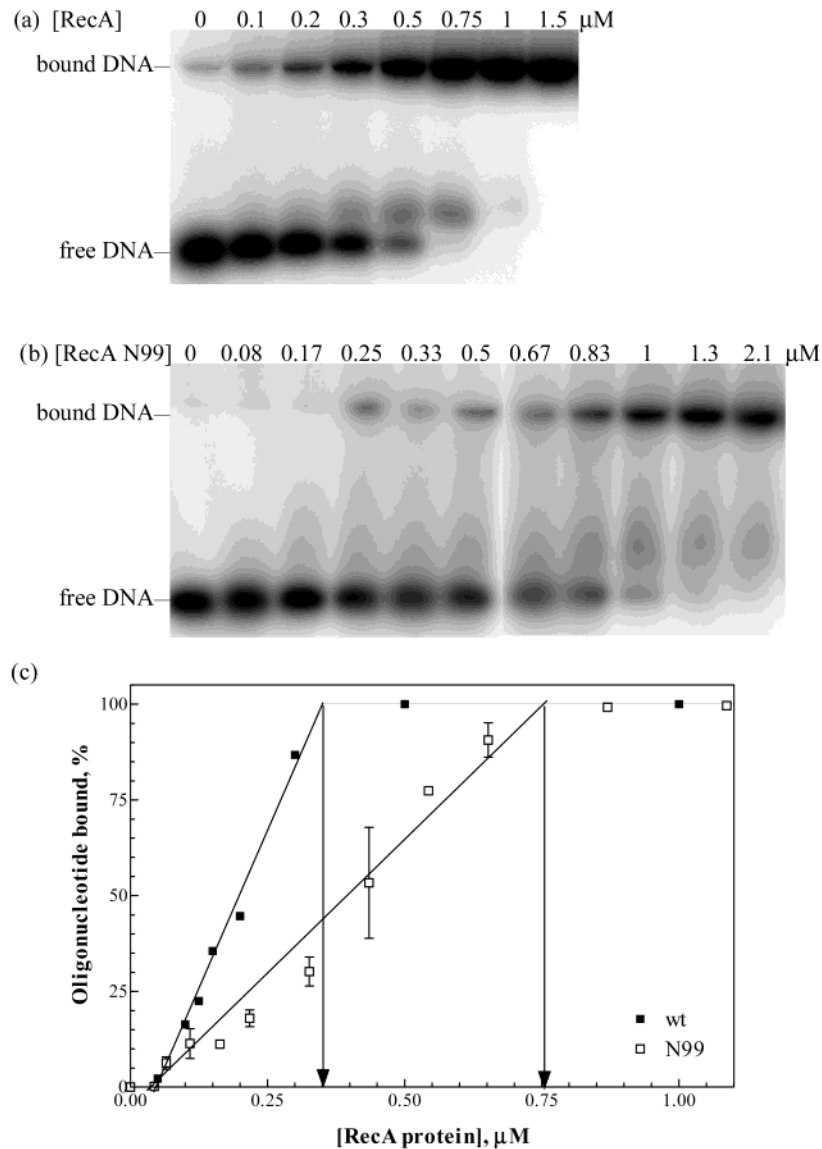
FIGURE 3: RecA N99 protein is unable to bind ssDNA oligonucleotide in the secondary DNA binding site. Incubation of ^{32}P-labeled oligonucleotide ssDNA (3 μM) with varying concentrations of RecA protein for 12 min was followed by an increase in the magnesium ion concentration and a further 10 min incubation. Reaction samples were run on a polyacrylamide gel, and the amount of unretarded DNA was quantified. The active concentration of RecA N99 protein is indicated in panels b and c. (a) Wild-type RecA protein, (b) RecA N99 protein, and (c) percentage of oligonucleotide shifted in gel plotted vs protein concentration. The failure of RecA N99 protein to bind a second equivalent of ssDNA is a reflection of its attenuated secondary DNA binding capacity. Panels a and b were created using Adobe Photoshop 5.0 to select the lanes of interest and to adjust the contrast for publication; quantification was performed using Image-QuaNT software from the original image.

observed for RecA N99 protein in the presence of ATP (data not shown); it is well-known that the affinity of RecA protein for DNA is higher in the presence of ATPγS than in the presence of ATP (*45*). Figure 7 shows the impaired ability of RecA N99 protein to catalyze the exchange of circular M13 ssDNA for the homologous strand in linear M13 dsDNA in the presence of ATPγS. Under these conditions, 57% of the dsDNA is taken up by wild-type RecA protein after 40 min to form joint molecules (lane 5). The level of uptake of dsDNA in the RecA N99 protein reaction is 31% after 40 min (lane 9), approximately half that of wild-type RecA protein. The impaired ability of RecA N99 protein to form joint molecules is consistent with the diminished level of cell survival following DNA damage (*22*). The extent of obligatory binding of dsDNA to the secondary site is, however, diminished compared to that for the wild-type protein, consistent with our DNA binding results. This

reduced level of joint molecule formation points to the involvement of L1 in the secondary DNA binding site.

RecA N99 Protein Catalyzes the Cleavage of the LexA Repressor. To investigate the constitutive coprotease phenotype of RecA N99 protein, LexA cleavage was assessed *in vitro*. RecA N99 protein is capable of catalyzing the autocleavage of the LexA repressor to approximately 60% of the extent of wild-type RecA protein with poly(dT) as the polynucleotide cofactor (Figure 8). The use of RNA as a polynucleotide cofactor was also investigated since it was found that RecA1202 and RecA1211 proteins could cleave the LexA repressor using rRNA or tRNA as a cofactor (*50*). No coprotease activity was measured for RecA N99 protein with poly(rU) (data not shown).

Since L1 was proposed to be both the LexA binding site and the secondary DNA binding site, the effect of excess polynucleotide on LexA repressor cleavage was examined
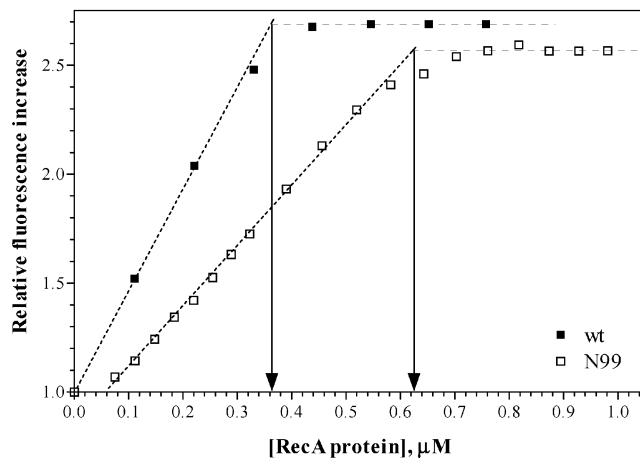
FIGURE 4: Binding of etheno M13 DNA to RecA N99 protein confirms the defect in secondary DNA binding. The change in the intrinsic fluorescence of etheno M13 DNA (3 μM) upon RecA protein binding was monitored. RecA protein was added at the indicated concentrations, whereas the active concentration of RecA N99 protein is indicated.

Table 1: Effect of SSB Protein on the Rate of ATP Hydrolysis[a]

	rate of ATP hydrolysis (μM/min)	
	without SSB	with SSB
wild-type RecA	13.1	31.5
RecA N99	10.1	7.48

[a] SSB protein (0.6 μM) was added to a reaction mixture of 3 μM M13 ssDNA and 1.5 μM RecA protein, after a steady state of hydrolysis had been reached.
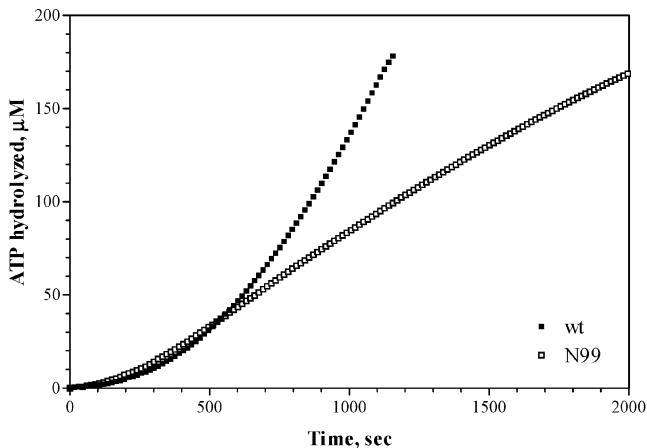

FIGURE 5: Displacement of SSB protein from ssDNA measured by ATP hydrolysis activity. SSB protein (0.6 μM) was allowed to bind to 3 μM M13 ssDNA for 5 min at 37 °C before the addition of 1.5 μM RecA protein under the standard ATP hydrolysis conditions. The lag times for the appearance of ATPase activity were determined by extending the linear portion of the trace, after a steady state of hydrolysis had been reached, back to zero and finding the point of intersection, as indicated.

with wild-type RecA protein and RecA N99 protein. Rehrauer et al. (25) observed that the addition of excess ssDNA was inhibitory to a LexA repressor cleavage reaction. Figure 8 shows the effect of excess poly(dT) on a preformed ternary complex of RecA protein, ATPγS, and poly(dT) on the LexA repressor cleavage. Both wild-type RecA protein and RecA N99 protein show a decrease in the extent of LexA repressor cleavage with the addition of excess poly(dT). The extent of wild-type RecA protein coprotease activity is reduced from
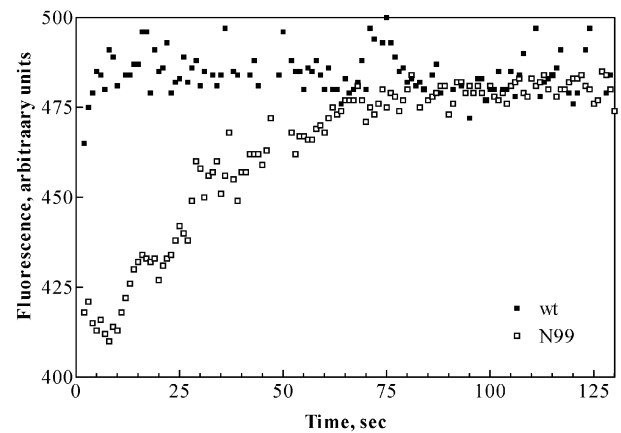

FIGURE 6: In contrast to wild-type protein, RecA N99 protein binds ssDNA slowly, showing a nucleation-limited lag. RecA protein (0.1 μM) was added to 3 μM etheno M13 DNA, and the change in fluorescence was measured over time.
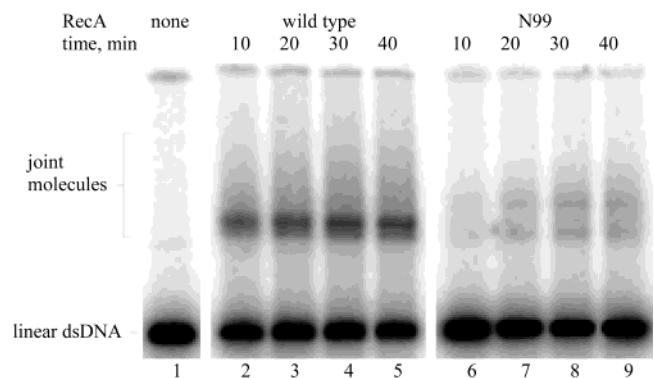

FIGURE 7: RecA N99 protein has diminished DNA strand exchange activity. M13 ssDNA (1.67 μM) and 0.15 μM SSB protein were incubated for 10 min followed by the addition of 1 mM ATPγS and 1 μM RecA protein. After a further 10 min incubation, the reaction was started with the addition of radiolabeled 1.67 μM linear M13 dsDNA; aliquots of the reaction mixture were removed at the specified times. Lane 1 shows a no protein reaction; lanes 2−5 show a reaction that included wild-type RecA protein, and lanes 6−9 show a reaction that included RecA N99 protein. Within each set of RecA protein lanes, from left to right, are 10, 20, 30, and 40 min time points. The figure was created using Adobe Photoshop 5.0 and Micrografx Designer to select the lanes of interest and to adjust the contrast for publication; the quantification was performed using Image-QuaNT software from the original image.

93 to 48%, and RecA N99 protein activity is reduced from 59 to 36% with the addition of a 20-fold excess of poly-(dT); the ability of each RecA protein to cleave the LexA repressor is reduced to approximately half of its level in the absence of excess poly(dT). ATPγS was used in this experiment to ensure that there was no redistribution of RecA protein upon addition of excess DNA, resulting in disruption of the original nucleoprotein filament. To confirm this, the stability of the original nucleoprotein complex was examined by gel electrophoresis using a radioactive DNA substrate and was found to be stable upon addition of excess poly(dT) (data not shown). The interference of excess DNA in the LexA repressor cleavage reaction suggests an active secondary DNA binding site in RecA N99 protein but, again, only in the presence of ATPγS. This interaction was detectable in this experiment because of the high affinity of RecA protein for poly(dT) (and the use of ATPγS), as compared to a lower affinity for oligonucleotide-length DNA, which precluded binding in the experiment shown in Figure 3.
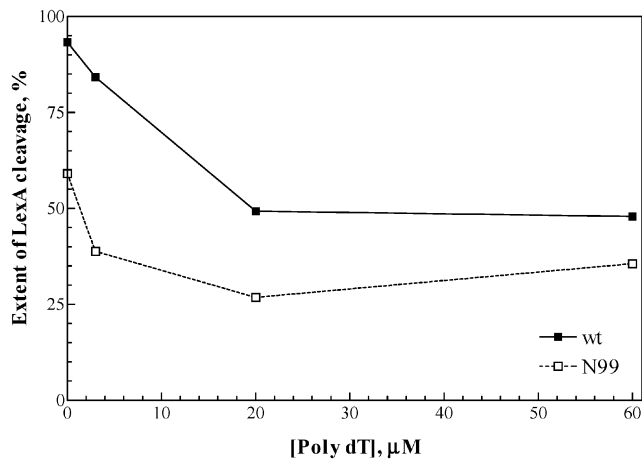
FIGURE 8: In the presence of ATPγS, excess poly(dT) inhibits LexA cleavage. As described in Experimental Procedures, poly(dT) was added at the indicated concentrations, along with 10 μM LexA repressor, to a complex of 3 μM poly(dT) and 1 μM RecA protein. The reaction was allowed to proceed for 20 min, and the reaction products were analyzed by polyacrylamide gel electrophoresis.

DISCUSSION

Mutations in the L1 region of the RecA protein were found to affect both coprotease and recombination activity (*22*); the mutant RecA N99 protein displays constitutive coprotease activity and significantly reduced recombinational repair activity *in vivo*. Our examination of the biochemical behavior of this mutant *in vitro* finds that it differs from wild-type RecA protein in several aspects; the primary difference is its DNA binding capabilities. RecA N99 protein can form an enzymatically active nucleoprotein filament as revealed by its ATP hydrolysis and LexA cleavage activity; however, interaction of the filament with the second DNA molecule necessary for DNA strand exchange is impaired. This may be the result of a change in the secondary DNA binding site which translates into a change in affinity for the second DNA molecule. This impaired binding ability of the secondary site is revealed by comparing the etheno DNA and oligonucleotide binding measurements to the DNA binding site size derived from ATP hydrolysis measurements. Wild-type RecA protein characteristically displays a 2-fold difference between DNA binding site sizes determined by the former two methods, which measure both primary and secondary site binding, and the site size obtained from ATPase activity measurements, which measures solely primary site binding (Figures 3 and 4 vs Figure 1) (*51, 52*). This difference is absent for RecA N99 protein, indicating that a second molecule of DNA is not bound or that binding is not observed by these assays. It is our interpretation that a second molecule of DNA is unable to interact with the nucleoprotein filament in the presence of ATP, as measured by these experiments. However, in the presence of ATPγS, where the affinity of RecA protein for DNA is increased (*45*), binding to the secondary site is detectable. This statement is substantiated by two lines of evidence: (1) the diminished, but not completely impaired, ability of RecA N99 protein to bind a second DNA molecule is indicated by the reduced level of joint molecule formation in a DNA strand exchange assay (Figure 7), and (2) the observed interference in LexA repressor cleavage activity by an excess of ssDNA. Both of these results were obtained with ATPγS as the nucleotide

cofactor. Since no binding to the secondary site could be measured in the presence of ATP, a reduction in the binding capability *in vivo* is also inferred. The reduced ability to bind a second DNA molecule and the consequent diminished DNA strand exchange activity are sufficient to explain the impaired recombination phenotype of RecA N99 protein. It is possible that RecA N99 protein is also defective in steps following synapsis; however, this could not be examined due to the absence of DNA strand exchange in the presence of ATP.

The ATP hydrolysis activity of this mutant is reduced by 63%. Also, the activity is not significantly altered by the addition of SSB protein to a preformed filament, whereas the activity measured for wild-type RecA protein increases due to the removal of inhibitory secondary structure by SSB protein (*38, 47, 48*). This indicates that although RecA N99 protein is not displaced by SSB protein, it is likewise not able to displace SSB protein and gain access to the full length of the DNA strand to form a contiguous filament. Another possibility is that RecA N99 protein is already fully coating the ssDNA, and since it is not displaced by SSB protein, no effect is measured. A comparison of the ATP hydrolysis activity with poly(dT) as the polynucleotide cofactor (Figure 1), which contains no secondary structure, and M13 ssDNA (Table 1) shows that the rate of ATP hydrolysis is only slightly increased in the former. This suggests that RecA N99 protein may be almost fully coating the M13 ssDNA in the absence of SSB protein.

The ability of RecA protein to displace SSB protein from ssDNA has been directly related to its rate of association with ssDNA (*18, 33, 53*). RecA430 protein displays both a slower rate of association with ssDNA than wild-type RecA protein and an impaired ability to displace SSB protein (*18*). An increased rate of association with ssDNA was measured for RecA730, RecA803, and RecA441 proteins, which correlates with their improved ability to displace SSB protein (*33*). RecA N99 protein was found to have a decreased rate of association and would be expected to be less competitive with SSB protein for ssDNA binding sites; however, compared to that of the wild-type protein, the characteristic lag before the onset of ATPase activity is not very different for RecA N99 protein. The foundation of this unexpected result is not understood; perhaps the cluster size of RecA N99 protein required to generate ATP hydrolysis activity is smaller than that for the wild-type protein. If this is the case, a smaller amount of bound RecA N99 protein would result in hydrolysis and, hence, a smaller amount SSB protein would need to be displaced, as compared to the amount of wild-type RecA protein. Regardless, RecA N99 protein clearly has altered ssDNA binding properties, suggesting an involvement of L1 in the primary DNA binding site, as well as the secondary site.

Another mutant RecA protein, RecA142, is also defective in genetic recombination (*54*). It was found that this protein was capable of binding ssDNA and has ssDNA-dependent ATP hydrolysis activity. However, RecA142 protein cannot form coaggregates between ssDNA and dsDNA, lacks joint molecule formation and DNA strand exchange activity, and is unable to compete with SSB protein for ssDNA binding. The foundation of the inability to bind the second molecule of DNA was found to be the result of either an alteration in the form of the high-affinity binding conformation or a

detrimental effect of the mutation on the interconversion between high- and low-affinity states (*31*). The site of mutation in RecA142 protein, Ile225 substituted with Val (*55*), is outside of L2, which is implicated in being part of the primary DNA binding site (*8*), and would be likely to alter the ssDNA properties, as shown by Kowalczykowski et al. (*31*). RecA N99 protein displays some altered ssDNA binding properties (i.e., attenuated ATP hydrolysis activity, no measurable level of ssDNA binding in the absence of cofactor, a decreased rate of association, and an altered competition with SSB protein). This suggests that the site of mutation has affected the conversion to the high-affinity state or the primary DNA binding site; this is in agreement with the cross-linking studies of Malkov and Camerini-Otero, who identified L1 and L2 as the primary DNA binding site (*14*), and Wang and Adzuma, who concluded L1 is the primary binding site (*15*). Additionally, L1 can be assigned as part of the secondary DNA binding site and LexA binding site (*8, 10*) which is substantiated by the impaired binding of a second DNA molecule and the reduction in DNA strand exchange activity of RecA N99 protein. It appears that L1 participates in both the primary and secondary binding sites.

The nature of the coprotease activity was examined to determine if alternate polynucleotide cofactors may lead to constitutive activity. RecA1202 protein and RecA1211 protein were shown to be active for LexA repressor cleavage with rRNA and tRNA as cofactors (*50*). RecA N99 protein did not have LexA repressor cleavage activity with poly-(rU) (data not shown), using either ATP or dATP, indicating that utilization of RNA was not responsible for the observed constitutive activity. RecA N99 protein was capable of LexA cleavage with poly(dT) and ATP or dATP in the reaction. There was also no ATP hydrolysis activity observed for RecA N99 protein with poly(rU) (data not shown) which points to the lack of filament formation on this substrate.

The mutually exclusive nature of RecA protein binding to a second DNA molecule or to the LexA repressor was examined by Rehrauer et al. (*25*). Addition of excess ssDNA was found to inhibit the cleavage of the LexA repressor, indicating that the LexA repressor and the second DNA molecule share the same or overlapping binding site. Although RecA N99 protein is able to cleave the LexA repressor in the presence of poly(dT) and ATP, a substitution of ATPγS was used to remove the potential for redistribution of the RecA protein from the original nucleoprotein filament to the excess poly(dT). We did not anticipate that the addition of excess polynucleotide would have an effect on RecA N99 protein since the binding of a second DNA molecule is impaired; however, the addition of ATPγS alters the DNA binding affinity and allows interaction in both the primary and secondary DNA binding sites. The data presented here suggest that the effect of the excess poly(dT) on the extent of LexA repressor cleavage is due to a competitive binding, as it is with wild-type RecA protein. Another explanation is that the affinity of this mutant protein for the LexA repressor is also diminished such that even the reduced amount of binding in the secondary site of which RecA N99 protein is capable inhibits LexA repressor binding.

The possibility of a reduced affinity for LexA repressor binding by RecA N99 protein does not lend itself to constitutive coprotease activity. A likely explanation for the constitutive activity is the buildup of ssDNA due to the lack of recombinational repair. The constitutive activity of RecA441 protein (*49*), RecA730 protein (*33*), and RecA P67W protein is due to an enhanced ability to displace SSB protein and, hence, to gain access to ssDNA in an undamaged cell. The ability of RecA N99 protein to displace SSB protein may also allow for access to ssDNA. A comparison of the displacement of SSB protein by RecA N99 protein or RecA803 protein with that of wild-type RecA protein reveals some similarity (*33*). As concluded for RecA803 protein, this level of SSB protein displacement is not sufficient for constitutive induction of the SOS response (*33*). The constitutive coprotease activity is probably the result of ssDNA generated by naturally occurring damage which persists in RecA N99 cells due to the significantly impaired ability to perform recombinational repair and, hence, leads to a buildup of ssDNA available as a cofactor for coprotease activity.

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