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Interaction of *Escherichia coli* RecA Protein with LexA Repressor

I. LexA REPRESSOR CLEAVAGE IS COMPETITIVE WITH BINDING OF A SECONDARY DNA MOLECULE*

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Essential to the two distinct cellular events of genetic recombination and SOS induction in *Escherichia coli*, RecA protein promotes the homologous pairing and exchange of DNA strands and the proteolytic cleavage of the LexA repressor, respectively. Since both of these activities require single-stranded DNA (ssDNA) and ATP, the inter-relationship between these reactions was investigated and found to display many parallels. The extent of active complex formed between RecA protein and M13 ssDNA, as measured by both ATP hydrolysis and LexA proteolysis, is stimulated in a similar manner by either a reduction in magnesium ion concentration or the presence of single-stranded DNA binding (SSB) protein. However, unexpectedly, SSB protein inhibits both LexA proteolysis and ATP hydrolysis (in assays containing repressor) at concentrations of RecA protein that are substoichiometric to the ssDNA, arguing that LexA repressor affects the competition between RecA and SSB proteins for limited ssDNA binding sites. Additionally, attenuation of LexA repressor cleavage in the presence of double-stranded DNA or by an excess of ssDNA suggests that interaction of the RecA nucleoprotein filament with either LexA repressor or a secondary DNA molecule is mutually exclusive. The significance of these results is discussed in the context of both the regulation of inducible responses to DNA damage, and the competitive relationship between the processes of SOS induction and genetic recombination.

Insults inflicted upon DNA represent a serious challenge to cellular survival in all living organisms. The RecA protein has several distinct roles in maintaining genetic integrity within *Escherichia coli*. In promoting the recognition and subsequent exchange of strands between homologous DNA molecules (for a recent comprehensive review see Ref. 1 and references therein), the RecA protein is central to recombinational repair events. In addition, the RecA protein coordinates the cellular response to factors that confer DNA damage or interfere with DNA replication by inducing the expression of a set of unlinked genes, *recA* included, comprising the SOS regulatory system (2–4). Initiation of the SOS response occurs when RecA protein becomes activated to stimulate cleavage of the LexA protein, the transcriptional repressor of SOS regulon genes (5). The LexA repressor is not the only target of activated RecA protein; cleavage of lytic repressors from various lambdoid bacteriophage accounts for prophage induction (6), while maturation of the mutagenesis factor (7), UmuD protein, is necessary for bypass of lesions during DNA replication (8). Repair of DNA damage and alleviation of blocks to replication serve to eliminate the signal that activates the RecA protein; consequently, as intact LexA repressor reaccumulates, the expression of SOS genes return to normal uninduced levels.

The ability to interact with DNA and a nucleotide triphosphate cofactor is fundamental to the remarkably diverse biochemical activities of the RecA protein. In a series of kinetically discernible steps, *in vitro*, the RecA protein promotes homologous pairing and transfer of strands between a variety of DNA substrates, provided one possesses some single-stranded character (1, 9). The elementary stage of the reaction involves assembly of a nucleoprotein complex, through the nonspecific and cooperative binding of the RecA protein to a fully or partially ssDNA[1] molecule, in the presence of ATP, that is capable of searching for and establishing homologous contacts with duplex DNA. Similarly, initial studies examining proteolysis of phage repressors by RecA protein also revealed a requirement for both nucleotide triphosphate and single-stranded polynucleotide (6, 10, 11). Cleavage of the LexA (12) and UmuD (13) proteins also occurs, *in vitro*, when RecA protein binds to ssDNA and either ATP, dATP, or the relatively non-hydrolyzable analogue, ATPγS. However, in contrast to a traditional protease, the RecA nucleoprotein filament plays an indirect role as a "coprotease" in stimulating the specific autodigestion of these target proteins (14, 15). The relative abundance of free RecA protein and ATP in uninduced cells implicates ssDNA as the critical component responsible for *in vivo* activation of the RecA protein. Consistent with this proposal, the production of single-stranded regions in damaged DNA through either the helicase activity of the RecBCD protein (16) or ongoing DNA replication (17) is a prerequisite for derepression of the SOS regulon by various inducing treatments. The role of ssDNA as

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[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ATPγS, adenosine 5′-[thio] triphosphate; SSB, single-stranded DNA binding; poly(dT), polydeoxy(thymidylic acid); etheno M13 ssDNA, modified M13 ssDNA containing 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine residues; IOD, integrated optical density.

the activating signal, *in vivo*, is confirmed by observations that infection by mutant filamentous phage that are defective in complementary (minus) DNA strand synthesis induces the SOS response (18).

The binding of RecA protein to ssDNA and ATP yields a ternary complex that is the functional species in both the homologous pairing of DNA and the proteolytic cleavage of the LexA repressor; since a parallel consequence of this binding is the hydrolysis of ATP, the formation and properties of the ternary complex can be monitored indirectly by measuring the ssDNA-dependent ATP hydrolysis activity of the RecA protein. To further our understanding of the mechanisms of and relationship between the cellular processes of SOS induction and genetic recombination, we have compared cleavage of the LexA repressor by this ternary complex to hydrolysis of ATP. As anticipated, considering the shared requirement for ssDNA and ATP, the LexA repressor cleavage and ATP hydrolysis activities of RecA protein display many parallels. The ability of SSB protein to stimulate maximal rates of RecA protein-promoted LexA proteolysis and ATP hydrolysis, *in vitro*, is consistent with it being genetically critical in both the response to and the recombinational repair of DNA damage (1, 19). Unexpectedly, however, demonstration that the LexA repressor influences the competition between RecA and SSB proteins for limited ssDNA binding sites identifies an additional manner by which inducible responses to DNA damage may be regulated. Despite many earlier studies focusing on either homologous DNA pairing or coprotease activities, it remained unclear whether proteolytic cleavage is independent of or competitive with other RecA protein-promoted reactions. Our characterization of the inhibition of LexA proteolysis by dsDNA and excess ssDNA provides direct biochemical evidence that supports the idea, based on electron microscopy (20), that interactions between the RecA nucleoprotein filament and either the LexA repressor or a secondary DNA molecule are mutually exclusive. Thus, the cellular processes of SOS induction and genetic recombination may be intrinsically competitive in nature. Studies using a non-cleavable mutant of the LexA repressor protein, presented in the accompanying paper (21), reinforce these views.

MATERIALS AND METHODS

Chemicals and Buffers

All chemicals were reagent grade; solutions were made using Barnstead NANOpure water. ATP, dATP, and ATPγS were purchased from Pharmacia Biotech, Inc., Sigma, and Boehringer Mannheim, respectively. The nucleotides were dissolved as concentrated stocks at pH 7.5, and their concentrations were determined spectrophotometrically using an extinction coefficient of 1.54×10^4 M^{-1} cm^{-1} at 260 nm.

Proteins

The RecA protein was purified from *E. coli* strain JC12772 using a modified preparative procedure[2] based on spermidine acetate precipitation (22). SSB protein was purified from *E. coli* strain RLM727 as described (23). Protein concentrations were determined using molar extinction coefficients of 2.7×10^4 M^{-1} cm^{-1} for RecA protein and 3.0×10^4 M^{-1} cm^{-1} for SSB protein, both at 280 nm. LexA repressor was purified from strain JL652 using essentially the protocol of Schnarr *et al.* (24) with the following modifications; phosphocellulose fractions containing LexA repressor were assayed to detect, and pooled to avoid, an overlapping elution of deoxyribonuclease activity; following dialysis, a step elution from a Q-Sepharose column using 100 mM NaCl resolved the intact LexA repressor (≥98% pure based on SDS-polyacrylamide gel electrophoresis) from a contaminant having DNA-dependent ATP hydrolysis activity. Concentration of the LexA repressor was determined using a molar extinction coefficient of 7300 M^{-1} cm^{-1} at 280 nm (25).

Lactate dehydrogenase and pyruvate kinase were both purchased from Sigma as ammonium sulfate suspensions; working solutions were prepared by centrifuging a homogeneous aliquot and resuspending the protein pellet in reaction buffer.

DNA

Single- and double-stranded DNA from bacteriophage M13mp7 and plasmid DNA from pBR322 were purified according to procedures outlined by Messing (26); duplex DNA from M13 bacteriophage replicative form ("homologous") and pBR322 ("nonhomologous") were linearized using *Eco*RI and *Nde*I restriction endonucleases, respectively. Molar nucleotide concentrations were determined using extinction coefficients of 8780 M^{-1} cm^{-1} for ssDNA and 6500 M^{-1} cm^{-1} for dsDNA, both at 260 nm. Etheno M13 DNA was prepared from viral DNA as described (27), while poly(dT) was purchased from Pharmacia Biotech, Inc. and dissolved as a concentrated stock using TE buffer (10 mM Tris-HCl and 1 mM EDTA (pH 7.5)); nucleotide concentrations were determined using molar extinction coefficients of 7000 and 8520 M^{-1} cm^{-1} at 260 nm, respectively.

LexA Repressor Cleavage Assay

Unless otherwise indicated, incubations and reactions were conducted at 37 °C in a standard buffer comprised of 25 mM Tris-hydrochloride (pH 7.5), 10 mM magnesium chloride, 50 mM sodium chloride, 0.1 mM dithiothreitol, and 1 mM ATP using the following order of addition: to standard buffer with the indicated amounts of magnesium chloride, sodium chloride, and nucleotide triphosphate cofactor (ATP, dATP, or ATPγS), and a regenerating system consisting of 8 mM phosphoenolpyruvate (Sigma) and 12.5 units/ml pyruvate kinase (25 units/ml for dATP), ssDNA (M13, poly(dT) or etheno M13), and RecA protein were incubated. When present, the SSB protein (0.25 μM) was added 5 min after the RecA protein, unless otherwise noted. All reactions were initiated with the addition of 10 μM LexA repressor. If included, either homologous or nonhomologous linear dsDNA was added immediately prior to the LexA repressor.

Cleavage of the LexA repressor by RecA protein was measured using 15% SDS-polyacrylamide gel electrophoresis as described previously (12, 28). The integrated optical density (IOD) of resolved bands corresponding to intact LexA repressor, the two proteolytic fragments, and the RecA protein were quantitated from Coomassie Brilliant Blue-stained gels using a Millipore Bioimage Imaging System. Lane-dependent artifacts due to gel loading or running were accounted for in a given reaction by normalizing the IOD values measured for both intact and proteolytic fragments of LexA repressor to that determined for the RecA protein. The extent of LexA repressor cleavage was calculated as a ratio of the normalized IOD value for the intact LexA repressor relative to the sum of each normalized IOD value for the intact LexA repressor and for the two proteolytic fragments. All extents of repressor cleavage are corrected for the amount of cleaved LexA repressor at zero time (≥85% intact). Cleavage rates were determined using the slope of a least squares fit of the initial linear portion of reaction time courses.

ATP Hydrolysis Assay

The single-stranded DNA-dependent hydrolysis of ATP was measured using a continuous spectrophotometric assay that couples ADP production to the oxidation of NADH (29) as adapted for use with the RecA protein (30). Assays were carried out under those indicated conditions used for measuring the rate of LexA proteolysis and included 0.2 mg/ml NADH (Sigma) and 12.5 units/ml lactate dehydrogenase. In reactions including repressor, the LexA repressor was added last to ongoing assays, and steady state rates of ATP hydrolysis were determined within regions of the time course where the LexA repressor remained at least 50% intact.

Both the rates of LexA proteolysis and ATP hydrolysis reported are the average of minimally two independently determined sets of data. Experimental errors in the rates of ssDNA-dependent LexA repressor cleavage and ATP hydrolysis were calculated to be less than or equal to ±9 and ±5%, respectively, of the average values.

RESULTS

Influence of Magnesium Ion on RecA Protein-stimulated Cleavage of LexA Repressor—In the presence of ATP, the binding of RecA protein to ssDNA is stabilized by magnesium ion (27). Moreover, characterization of the ssDNA-dependent ATP hydrolysis activity of the RecA protein demonstrated that the magnesium ion concentration affects the extent of RecA nucleoprotein filament formation (30). Maximum rates of ATP hydrolysis are normally observed at a ratio of one RecA protein

[2] S. C. Kowalczykowski, manuscript in preparation.

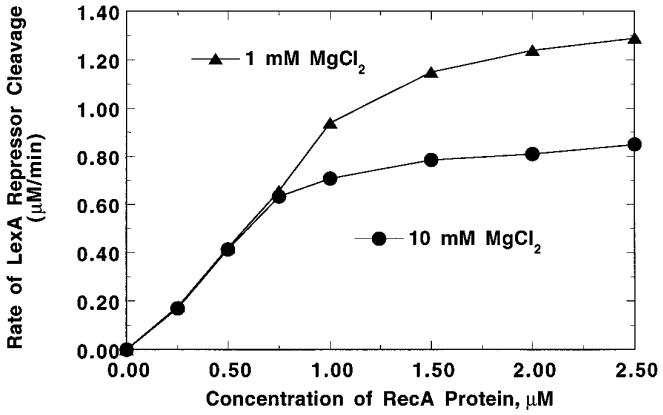

FIG. 1. **The influence of magnesium ion concentration on LexA repressor cleavage.** All assays were performed in standard reaction buffer (see "Materials and Methods") using 4.4 μM (nucleotides) M13 ssDNA with the RecA protein concentration varying as indicated. *Triangles* and *circles* represent reactions containing 1 or 10 mM magnesium chloride, respectively.

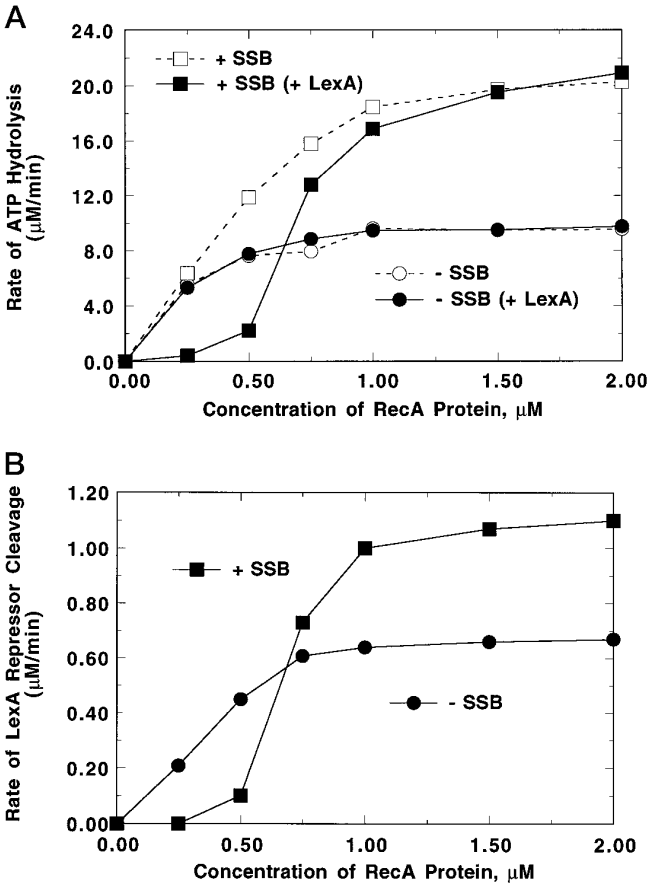

FIG. 2. **The effect of SSB protein on both ATP hydrolysis and LexA repressor cleavage.** *A,* represents the RecA protein concentration dependence of M13 ssDNA-dependent ATP hydrolysis activity, whereas *B* indicates the rate of LexA proteolysis obtained as a function of the RecA protein concentration. Standard buffer conditions for each of the assays were employed (see "Materials and Methods") using 3 μM (nucleotides) M13 ssDNA in the absence (*circles*) or presence (*squares*) of a saturating amount of SSB protein (0.25 μM). Reactions monitoring the rate of ATP hydrolysis, performed in the presence of 10 μM LexA repressor, are indicated by *solid lines*.

monomer per three nucleotides of ssDNA. However, secondary structure intrinsic to ssDNA is stabilized by elevated levels of magnesium ion and prohibits RecA protein from saturating the ssDNA (31); consequently, this results in apparently lower maximal rates of ATP hydrolysis and higher binding stoichiometries (30). For this reason, we examined the influence of magnesium ion on LexA repressor cleavage as a function of RecA protein concentration at a fixed amount of M13 ssDNA (Fig. 1). Over an initial range of RecA protein concentration (<0.75 μM), the rates of LexA repressor cleavage are equivalent at either 1 or 10 mM magnesium chloride and increase linearly with respect to the amount of RecA protein. At 10 mM magnesium chloride, proteolytic activity saturates at 0.8 μM/min, yielding an apparent site size of approximately five nucleotides of ssDNA per monomer of RecA protein. However, at 1 mM magnesium chloride, the level at which LexA repressor cleavage plateaus is enhanced (~1.35 μM/min) and the apparent stoichiometry is decreased to three to four nucleotides/RecA protein monomer. Both of these observations are consistent with the idea that destabilization of secondary structure at reduced magnesium chloride concentrations allows RecA protein to access more ssDNA, thereby resulting in a greater extent of ternary complex. Furthermore, the apparent first order rate constant for LexA proteolysis, as determined from the data at sub-saturating RecA protein concentrations, is virtually the same (k_{cat} ~0.92 min^{-1}) at either 1 or 10 mM magnesium chloride, confirming that the activity directly reflects the amount of active RecA protein ternary complex formed. In support of the critical requirement for ssDNA in activation of RecA protein during SOS induction, the rates of LexA repressor proteolysis were approximately 10-fold lower in the presence of either linear M13 or pBR322 dsDNA (data not shown).

Effect of SSB Protein on LexA Repressor Cleavage and ATP Hydrolysis Activities—Due to a preferential affinity for ssDNA, SSB protein disrupts secondary structure within native ssDNA and, upon being subsequently displaced, allows RecA protein to polymerize on these normally inaccessible regions (30–32). Thus, despite being a competitor for limited binding sites, the SSB protein indirectly contributes to the formation of a contiguous RecA nucleoprotein filament on ssDNA (30, 33). As illustrated in Fig. 2*A* (*dashed lines*), this effect of the SSB protein is manifest as a stimulation in the ATP hydrolysis activity of the RecA protein when M13 ssDNA is used as a substrate at elevated magnesium ion concentrations (28, 30). Before binding sites on M13 ssDNA become limiting, the steady-state rate of ATP hydrolysis increases linearly with RecA protein concentration in a manner unaffected by SSB protein; however, at saturating concentrations of RecA protein, the maximal rate of ATP hydrolysis achieved in the presence of SSB protein (~21 μM/min) is approximately 2-fold greater than that observed in its absence (~10 μM/min). In quantitative agreement with the enhanced rate at which ATP hydrolysis plateaus, there is a corresponding decrease in the apparent ssDNA binding stoichiometry derived for the RecA protein from ~six to ~three nucleotides of ssDNA per protein monomer. These findings argue that in the presence of SSB protein more of the M13 substrate is available to support the ssDNA-dependent ATP hydrolysis activity of RecA protein.

To assess the effect of SSB protein on LexA repressor digestion, proteolysis was examined under conditions identical to those used for ATP hydrolysis. As observed for ATP hydrolysis (Fig. 2*A*), inclusion of SSB protein enhances the rate at which proteolytic cleavage saturates (~1.15 μM/min) relative to reactions in which it was omitted (~0.65 μM/min) (Fig. 2*B*). Furthermore, the need for 1.5- to 2-fold higher RecA protein concentrations to achieve maximal rates of both LexA repressor cleavage and ATP hydrolysis in the presence of SSB protein indicates that the SSB protein enables the RecA protein to utilize the native ssDNA more completely. These results are consistent with the interpretation that by removing normally

inaccessible regions of secondary structure from native ssDNA, SSB protein facilitates formation of the RecA protein ternary complex and, consequently, stimulates those biochemical activities dependent on it (*i.e.* ATP hydrolysis, LexA repressor cleavage).

However, in contrast to ATP hydrolysis, LexA proteolysis is attenuated at sub-stoichiometric concentrations of RecA protein ($\leq$0.50 μM) in reactions containing SSB protein (Fig. 2*B*). To determine the origin of this reduction in cleavage activity, the hydrolysis of ATP was also examined in the presence of LexA repressor (Fig. 2*A, solid lines*). Addition of the LexA repressor to reactions containing SSB protein results in a decrease in ATP hydrolysis at less than stoichiometric concentrations of the RecA protein, similar to that observed in the proteolysis assay. This inhibition of RecA protein activity is SSB protein-dependent as the LexA repressor has no effect on ATP hydrolysis in the absence of SSB protein (Fig. 2*A*). Furthermore, optimal inhibition of the ssDNA-dependent activities of the RecA protein caused by the LexA repressor requires saturating amounts of SSB protein (*i.e.* either reducing the SSB protein concentration or increasing the M13 ssDNA concentration diminished the relative amount of inhibition, data not shown). Collectively, these observations indicate not only that the observed inhibition is a direct result of RecA protein being supplanted from the ssDNA by SSB protein but that the LexA repressor acts in an auxiliary manner to bring about this replacement of the RecA protein-ATP-ssDNA complex.

Stability of the Ternary Complex in the Presence of LexA Repressor—Similar to most protein-nucleic acid complexes (34), the stability of RecA protein-ssDNA complexes decreases with increasing salt concentrations (27). However, this sensitivity to disruption by salt is not due to competitive binding effects associated with cation displacement from the ssDNA phosphate backbone but rather is the result of anion displacement from the RecA protein during complex formation (35). Consequently, interactions between LexA and RecA proteins may destabilize the RecA protein-ATP-ssDNA ternary complex and thus account for the repressor-dependent increase in the inhibition of RecA protein activities by SSB protein. Since ATP hydrolysis and LexA proteolysis require formation of the same ternary complex, to address this possibility the salt sensitivities of these ssDNA-dependent activities of RecA protein were examined in parallel. As shown in Fig. 3, increasing amounts of sodium chloride cause a decrease in the ability of the RecA protein to promote either the hydrolysis of ATP or the cleavage of LexA repressor, in both the absence and presence of SSB protein. The concentrations of sodium chloride resulting in 50% inhibition of ATP hydrolysis and LexA proteolysis in the absence of SSB protein are similar (150 and 170 mM, respectively); in assays containing SSB protein, 250 mM sodium chloride is necessary for this degree of inhibition of hydrolysis, compared with 225 mM for proteolysis. In the case of either activities, SSB protein increases the apparent salt resistance by enhancing the formation of a more complete nucleoprotein filament. Furthermore, the amounts of sodium chloride required to reduce ATP hydrolysis by RecA protein to half-maximal levels, in either the absence or presence of SSB protein, are relatively unaffected by LexA repressor (Fig. 3*A*). Thus, based on the comparable salt sensitivities for these M13 ssDNA-dependent activities of the RecA protein, the LexA repressor does not drastically alter the steady-state stability of the RecA protein-ATP-ssDNA complex.

NTP Cofactor Binding by RecA Protein in the Presence of LexA Repressor—The stability and structure of the RecA nucleoprotein complexes are modulated by interaction with nucleotide cofactors. Binding of ATP increases the equilibrium

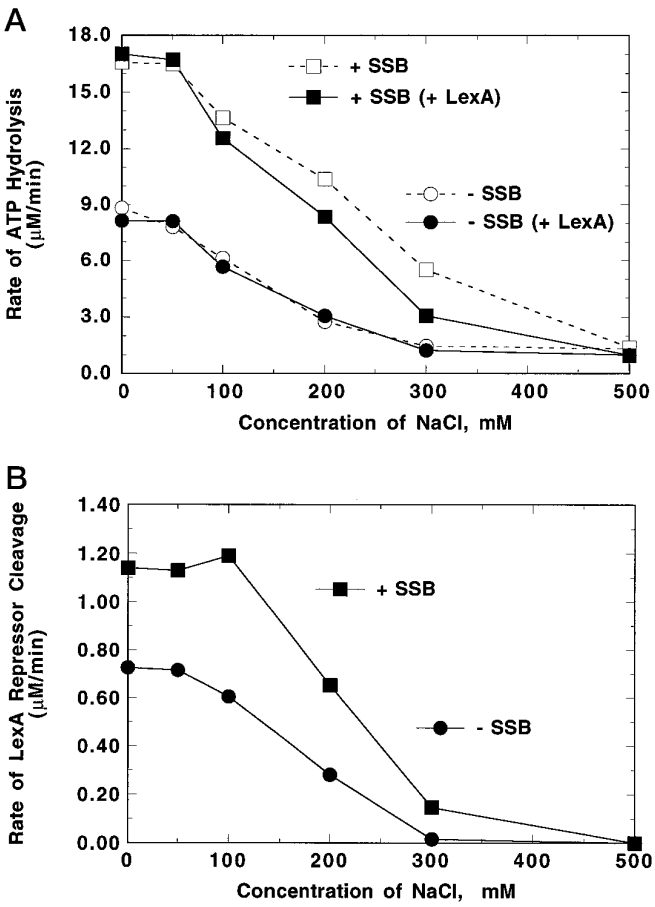

FIG. 3. **The effect of the NaCl concentration on both the ssDNA-dependent ATP hydrolysis and LexA repressor cleavage activities of RecA protein.** *A* and *B*, respectively, represent the sensitivity of the ssDNA-dependent ATP hydrolysis and LexA repressor cleavage activities of the RecA protein to increasing concentrations of sodium chloride. Assays were conducted as described under "Materials and Methods" with 1.5 μM RecA protein, 3 μM (nucleotides) M13 ssDNA, in either the absence (*circles*) or presence (*squares*) of the SSB protein using standard buffer conditions at the indicated concentrations of NaCl. ATP hydrolysis reactions performed in the presence of 10 μM LexA repressor are indicated by *solid lines*.

affinity of the RecA protein for ssDNA (27) and yields an extended conformation of the RecA protein-ssDNA complex (36–38); besides being fundamental to certain enzymatic activities, the high affinity ssDNA-binding state induced by ATP is required for RecA protein to be able to compete with, and displace, the SSB protein (9). In order to investigate the effects of repressor on the interaction of RecA protein with nucleotide cofactors, and to compare the requirements for nucleotide in ATP hydrolysis and LexA proteolysis, each of these ssDNA-dependent activities was examined as a function of the nucleoside triphosphate concentration (Fig. 4). As has been shown by others (39–41), the ATP hydrolysis activity of RecA protein is sigmoid with respect to nucleotide concentration. The ATP concentration needed to achieve half-maximal rates of hydrolysis defines an apparent K_m of 120 $\pm$ 4 μM for the RecA protein (Fig. 4*A*), which is similar to previously reported values (39–41). While maximal rates of ATP hydrolysis attained are unchanged, the presence of the LexA repressor shifts the apparent K_m of the RecA protein to a slightly higher ATP concentration (160 $\pm$ 8 μM). Furthermore, inclusion of repressor partially inhibits the M13 ssDNA-dependent ATP hydrolysis activity of the RecA protein at ATP concentrations between 60 and 200 μM (Fig. 4*A*).

In agreement with earlier characterizations (6, 10, 12, 42–

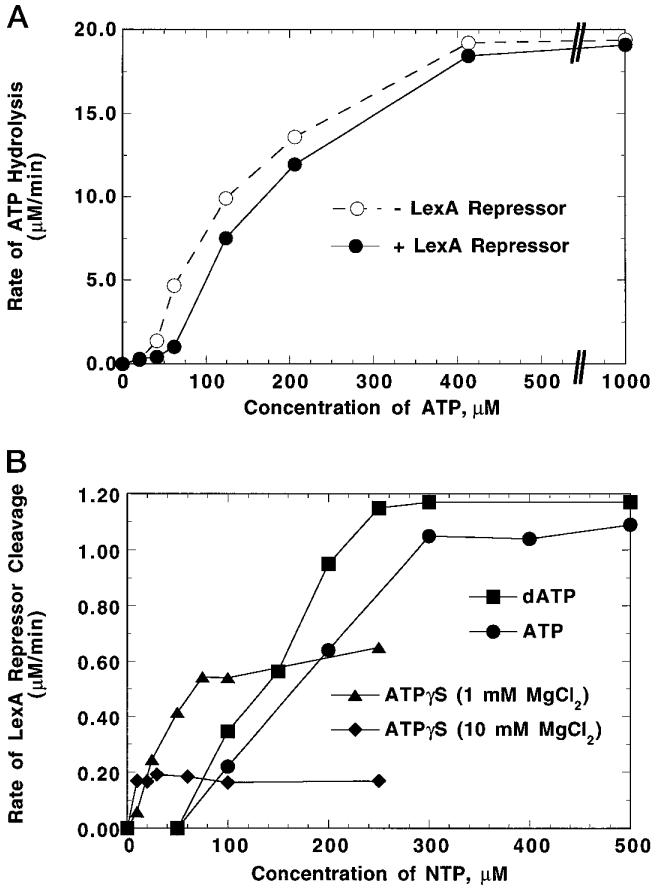

FIG. 4. **The nucleoside triphosphate concentration dependence of ssDNA-dependent ATP hydrolysis and LexA repressor cleavage activities of RecA protein.** The hydrolysis of ATP (*A*) and cleavage of the LexA repressor (*B*) by RecA protein (1 μM) was monitored under standard reaction conditions (see "Materials and Methods") using 7 μM (nucleotides) M13 ssDNA and either ATP (*circles*), dATP (*squares*), or ATPγS (*diamonds*). The concentration of the various nucleoside triphosphate (*NTP*) cofactors was varied as indicated. ATP hydrolysis was assayed in both the absence (*dashed lines*) and presence (*solid lines*) of 10 μM LexA repressor. Proteolysis reactions carried out with ATPγS at 1 mM magnesium chloride are represented by (*triangles*).

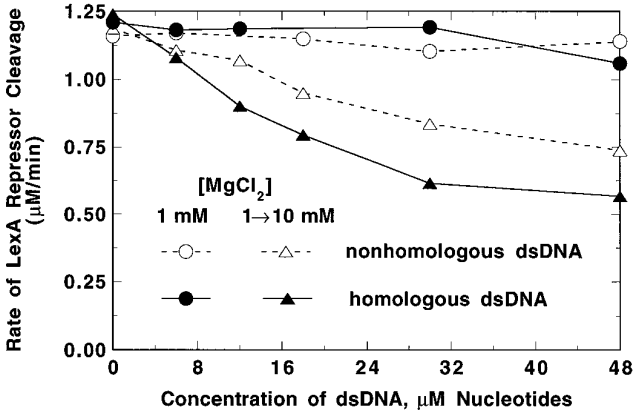

FIG. 5. **Effect of dsDNA on LexA repressor proteolysis by the RecA protein-ATP-ssDNA ternary complex.** In all reactions, the ternary complex consisting of RecA protein (1.5 μM), ATP, and M13 ssDNA (3.0 μM, nucleotides) was formed under standard buffer conditions (see "Materials and Methods") in the presence of 1 mM magnesium chloride. Either linear M13 ("homologous," *solid lines*) or pBR322 ("nonhomologous," *dashed lines*) dsDNA was added to reactions, in either the absence (*circles*) or presence (*triangles*) of additional magnesium chloride (final concentration of 10 mM), prior to initiating assays with LexA repressor.

44), ATP, dATP, and the essentially non-hydrolyzable analogue ATPγS support formation of RecA protein ternary complex that is active in proteolysis (Fig. 4*B*). Thus, the free energy associated with NTP binding allosterically induces the active form of the ternary complex that is crucial for proteolysis, whereas NTP hydrolysis, while it may be concurrent with, is not obligatory for the coprotease activity of the RecA protein. Nevertheless, under standard buffer conditions of 10 mM magnesium chloride, maximal rates of LexA repressor digestion observed with ATPγS are approximately 6-fold lower than those obtained with either ATP or dATP. However, as evidenced by the 3-fold increase in the rates of LexA repressor digestion observed at 1 mM magnesium chloride (Fig. 4*B*), proteolytic activity in the presence of ATPγS and stoichiometric amounts of M13 ssDNA is strongly dependent on magnesium ion concentration. The apparent K_m values derived from LexA proteolysis are 190 μM for ATP, 150 μM for dATP, and $\leq$30 μM for ATPγS. While consistent with the hierarchy in nucleotide binding affinity (ATPγS > dATP > ATP) previously established for the RecA protein (40), these concentrations needed to stimulate half-maximal rates of LexA proteolysis are 1.5–3-fold higher, in the cases of ATP and dATP, than the amounts necessary for nucleoside triphosphate hydrolysis (39, 40, 41, Fig. 4*A*). This requirement for higher concentrations of nucleotide cofactor for the coprotease activity of RecA protein, relative to hydrolysis, has also been observed in cleavage of lambda cI repressor (43).

Inhibition of ssDNA-dependent LexA Proteolysis Activity by dsDNA—RecA protein promotes the homologous pairing and subsequent exchange of strands between DNA molecules (45–48). The most extensively studied substrate pair are circular ssDNA and linear duplex DNA molecules. Just as in LexA repressor cleavage and ATP hydrolysis, the active species in the DNA pairing reaction is a RecA nucleoprotein filament assembled on ssDNA in the presence of ATP. While maximal rates of LexA proteolysis (Fig. 1) and ATP hydrolysis (30) using M13 ssDNA are observed at reduced concentrations (1 mM) of magnesium ion, the ability of RecA protein to promote the homologous pairing of DNA requires elevated levels (>1 mM) of magnesium ion (46, 49, 50). However, formation of a stoichiometric RecA nucleoprotein filament that is functional in DNA strand exchange can be accomplished by first allowing RecA protein to bind ssDNA at 1 mM magnesium, followed by a subsequent increase to higher magnesium concentrations (31). To understand the relationship between proteolytic cleavage and DNA pairing reactions, the effect of linear dsDNA on the coprotease activity of the RecA protein was investigated. At 1 mM magnesium chloride, addition of dsDNA has no effect on the rate of LexA repressor cleavage, whereas a shift in magnesium ion from 1 to 10 mM prior to initiating the assay with LexA repressor reduces proteolytic activity in a manner dependent on linear dsDNA concentration (Fig. 5). Inhibition by dsDNA is relatively independent of sequence, as nonhomologous dsDNA also reduces proteolytic cleavage of the LexA repressor (Fig. 5). These findings suggest that binding of dsDNA and LexA repressor by the RecA protein-ATP-ssDNA complex is competitive. Furthermore, the fact that the reduction in ssDNA-dependent proteolysis of the LexA repressor by dsDNA is contingent on magnesium ion concentrations that support homologous pairing also implies that this competition involves a site on the RecA nucleoprotein filament that participates in DNA strand exchange.

Inhibition of LexA Cleavage by Excess ssDNA—Although saturating amounts of ssDNA are required for optimal proteolytic cleavage, the presence of excess ssDNA has been previously reported to inhibit the coprotease activity of the RecA protein (10, 42, 44, 51, 52). The suggestion that interaction of the LexA

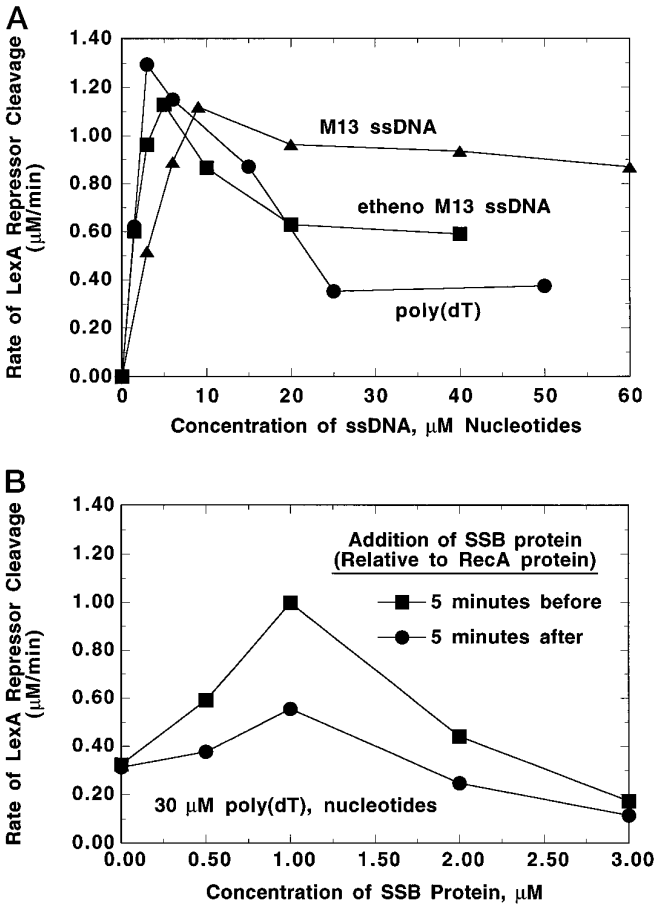

FIG. 6. **Effects of excess ssDNA on RecA protein coprotease activity in the absence and presence of SSB protein.** All reactions were conducted using 1 μM RecA protein in standard buffer conditions (see "Materials and Methods"). *A* represents the dependence of LexA repressor cleavage on the nucleotide concentration of ssDNA, M13 ssDNA (*triangles*), etheno M13 ssDNA (*squares*), and poly(dT) (*circles*). *B* demonstrates the effect of SSB protein on the proteolytic cleavage of LexA repressor under conditions of excess ssDNA; the concentration of poly(dT) in all assays was 30 μM (nucleotides). The amount of SSB protein was varied as indicated and was added to reactions containing poly(dT) either 5 min before (*squares*) or after (*circles*) the RecA protein.

repressor and dsDNA with the RecA protein nucleoprotein filament is a mutually exclusive event was an incentive to examine more carefully LexA repressor cleavage as a function of both the concentration and type of ssDNA. Maximal rates of both ATP hydrolysis (40, 41, Figs. 1 and 2*A*) (Figs. 1 and 2*A*) and LexA proteolysis (Fig. 6*A*) are observed at ratios ranging from three to seven ssDNA nucleotides/RecA protein monomer in the presence of native M13 ssDNA, etheno M13 ssDNA, or poly(dT). In direct contrast to ATP hydrolysis activity (data not shown), rates of LexA repressor cleavage decrease as the concentration for each ssDNA exceeds an optimal stoichiometry, with maximal inhibition of proteolysis being at ssDNA:RecA protein ratios of ~25 for both etheno M13 ssDNA and poly(dT), and an estimated 35–40 for M13 ssDNA. The higher apparent stoichiometries for both stimulation and inhibition of coprotease activity with M13 ssDNA are most likely due to the existence of DNA secondary structure. These low protein to ssDNA nucleotide ratios (~1:30), at which only 10–15% of the ssDNA is bound, parallel conditions that are optimal for the aggregation and renaturation of ssDNA molecules by RecA protein (53, 54). Unlike the addition of dsDNA, inhibition of coprotease activity by excess poly(dT) is not dependent on the magnesium ion concentration.[3] Nevertheless, these results are consistent with interaction of a secondary DNA molecule, whether single or double-stranded, being competitive with LexA repressor binding. Moreover, the extent of inhibition elicited by the various types of ssDNA (Fig. 6*A*) corresponds to the ssDNA binding affinity of RecA protein (poly(dT) > etheno M13 ssDNA > M13 ssDNA) (55), further supporting the idea that the binding of an additional ssDNA molecule by the RecA protein-ATP-ssDNA complex is responsible for the reduction in proteolytic cleavage of the LexA repressor.

The effect of SSB protein on the proteolytic cleavage activity of RecA protein in the presence of an excess of poly(dT) was also investigated. SSB protein alleviates the inhibition of RecA protein-mediated proteolysis that is caused by a surplus of polynucleotide (Fig. 6*B*). Under the conditions used for cleavage assays (10 mM magnesium chloride, 50 mM sodium chloride, and low protein binding density), the SSB protein would predominantly assume a site size of 65 ssDNA nucleotides/protein tetramer (56, 57). Thus, in the presence of 30 μM (nucleotides) poly(dT) (Fig. 6*B*), approximately 1.7 μM SSB protein would be required to leave 3 μM polynucleotide free to the RecA protein for complex formation; 3 μM is the amount of poly(dT) necessary for optimal repressor cleavage (~1.30 μM/min) at this RecA protein concentration (Fig. 6*A*). Fig. 6*B* shows that 1.0 μM SSB protein restores 75% (~0.98 μM/min) of the maximal coprotease activity of the RecA protein in the presence of 30 μM poly(dT). Rates of LexA repressor cleavage diminish dramatically as concentrations of SSB protein capable of completely saturating the polynucleotide (~2 μM) are approached (Fig. 6*B*). Since there is a reasonable correlation between the amount of SSB protein required to alleviate the inhibition of LexA repressor digestion by excess ssDNA, binding of polynucleotide by SSB protein must serve to sequester the surplus. Finally, the fact that SSB protein must be added prior to the RecA protein in order to see the greatest stimulatory effect (Fig. 6*B*) is consistent with the interpretation that efficient and stable uptake of additional poly(dT) molecules by a RecA protein-ATP-poly(dT) ternary complex is directly responsible for the inhibition in proteolytic cleavage of the LexA repressor.

DISCUSSION

To fulfill its respective roles in genetic recombination and in the induction of cellular responses to DNA damage, the multifunctional RecA protein promotes the homologous pairing of DNA and the proteolytic inactivation of the transcriptional LexA repressor. Despite being biochemically distinct, these activities have a common prerequisite, assembly of an active nucleoprotein complex through the binding of RecA protein to both ssDNA and ATP. Formation of the RecA protein-ATP-ssDNA complex can be most easily characterized, albeit indirectly, through measurement of the ssDNA-dependent ATP hydrolysis activity of the RecA protein. In this study, we have examined the proteolytic cleavage of the LexA repressor by this ternary complex relative to ATP hydrolysis to gain further insight into the mechanism of, and relationship between, the processes of genetic recombination and SOS induction.

As anticipated, given the mutual need for ATP and ssDNA, LexA repressor cleavage and ATP hydrolysis activities of the RecA protein share numerous similarities. When compared under identical conditions, proteolytic cleavage and ATP hydrolysis increase and saturate as a function of RecA protein concentration in a parallel manner (Figs. 1 and 2). Addition of SSB protein results in a 2-fold enhancement in the maximal rates of both ATP hydrolysis and LexA repressor cleavage,

[3] W. M. Rehrauer and S. C. Kowalczykowski, unpublished observations.

along with a corresponding decrease in the apparent binding stoichiometry for the RecA protein to three nucleotides of M13 ssDNA per protein monomer (Fig. 2). Equivalent levels of ATP hydrolysis (30) and LexA proteolysis (Fig. 1) by the RecA protein can be achieved in the absence of SSB protein at the same M13 ssDNA stoichiometry under conditions (*i.e.* low magnesium ion concentrations) that destabilize intramolecular base pairing inherent to the native ssDNA. Thus, the ssDNA binding activity of the SSB protein indirectly enhances activities of the RecA protein by allowing for a greater extent of ternary complex formation through the disruption of regions within native ssDNA that are normally inaccessible due to secondary structure (31). These results are consistent with, and provide a molecular basis for, the previous observation that SSB protein increases both the initial rate and extent of lambda cI repressor cleavage when concentrations of RecA protein were in excess of the ssDNA (58, 59). Analogous to both ATP hydrolysis (39–41) (Fig. 4*A*) and digestion of the lambda cI repressor (43), LexA proteolysis by the RecA protein ternary complex is sigmoid with respect to nucleotide cofactor concentration (Fig. 4*B*), suggesting that a critically sized polymer of RecA protein molecules contiguously bound to both NTP and ssDNA is the active species (40). This cooperative dependence on nucleotide, along with the stimulatory effects of SSB protein, and the optimal binding stoichiometry derived for the RecA protein are attributes not solely confined to the ssDNA-dependent activities of ATP hydrolysis and repressor cleavage but are fundamental properties also exhibited by RecA protein in the homologous pairing of DNA (for review see Ref. 9 and references therein). Consequently, despite the fact that the biochemical activities of the RecA protein that are essential to genetic recombination and SOS induction are remarkably different, they are dependent on the formation of essentially the same nucleoprotein filament.

As a consequence of being a diverse physiological response, induction of the SOS system is regulated by many factors. At the most elementary level, the extent of ssDNA (*i.e.* activating signal) produced at damaged sites and its endurance directly affect both the amounts of activated RecA protein and functional LexA repressor. The capacity of SSB protein to both protect and maintain ssDNA is consistent with deficiences in the *ssb* gene adversely affecting not only genetic recombination and the recombinational repair of DNA but also the cellular responses to DNA damage (for review see Ref. 1, 19). In this context, the ability of SSB protein to uniformly enhance the ssDNA-dependent ATP hydrolysis, homologous DNA pairing, and coprotease activities of the RecA protein is relevant. However, the demonstration of SSB protein-dependent inhibition of LexA proteolysis and ATP hydrolysis, in the presence of repressor (Fig. 2), indicates that the SSB protein and LexA repressor can also act in concert to inhibit RecA protein-promoted activities. Since inhibition is only observed at RecA protein concentrations that are sub-stoichiometric relative to the ssDNA (Fig. 2), the effects of the LexA repressor are most likely mediated through interaction with RecA protein. Additionally, because the observed inhibition is not only contingent upon, but requires, amounts of SSB protein sufficient to saturate the ssDNA present (data not shown), it must directly result from SSB protein replacing RecA protein on the ssDNA and preventing its reassociation to form an active ternary complex. While it has been established that the competitive binding of the RecA and SSB proteins to ssDNA depends on a number of experimental variables and solution conditions (33), these studies implicate the LexA repressor as an "effector" that is capable of altering competitive balance between RecA and SSB proteins for binding to limited ssDNA sites. Consequently, in making RecA protein more susceptible to challenge by SSB protein, the LexA repressor is capable of regulating the induction of the SOS response by perturbing the formation of a ternary complex responsible for its own proteolytic inactivation. Consistent with this proposal, while SSB protein deficiences cause extreme sensitivity to DNA damaging agents and high levels of DNA degradation, cells overproducing the SSB protein have also been reported to be moderately sensitive to ultraviolet irradiation (60, 61), suggesting that high levels of SSB protein interfere with activation of the RecA protein. These inhibitory effects of SSB protein overproduction on the induction of some SOS genes can be overcome by increasing levels of inducing agents (62), an *in vivo* result that parallels our *in vitro* suppression of SSB protein-dependent inhibition by excess M13 ssDNA.

The outcome of the competition between RecA and SSB proteins is determined by factoring in the relative binding affinities of the two proteins for ssDNA, along with their respective rates of association and dissociation. The delicate nature of this competitive balance and its impact on RecA protein function is illustrated in the characterization of two SSB mutant variants. Strains carrying either *ssb*-1 or *ssb*-113 alleles display similar deficiences in SOS induction and genetic recombination (63); however, the biochemical basis for these defects correspond to very different ssDNA binding properties of the mutant proteins. Due to a lower affinity for ssDNA, the SSB-1 protein is less effective at destabilizing base pairing within duplex DNA (64), possibly rendering it unable to facilitate RecA nucleoprotein filament formation. Conversely, inhibition of lambda cI repressor cleavage by SSB-113 protein (58) is most likely attributed to tighter ssDNA binding and the resultant displacement of the RecA protein (63). Consequently, the most reasonable explanation for a heightened susceptibility of RecA protein to inhibition by SSB protein in the presence of the LexA repressor is that interaction with LexA repressor decreases the steady state affinity of RecA protein for ssDNA. The sensitivities of LexA proteolysis and ATP hydrolysis, with or without repressor, to increasing concentrations of sodium chloride are similar (Fig. 3), a result that does not support this expectation. However, since the binding of ATP increases the affinity of RecA protein for ssDNA (27), and *vice versa* (40), slight differences in ternary complex stability might be manifest as changes in the apparent affinity for nucleotide cofactor. Examination of ATP hydrolysis and LexA proteolysis as a function of nucleotide concentration suggests that 2-fold higher concentrations of ATP are required for formation of an active RecA protein ternary complex in the presence of repressor (Fig. 4). These results may suggest that the LexA repressor alters ATP binding by RecA protein and in doing so may cause it to form a ternary complex that is less able to compete with SSB protein.

A more extensive investigation of the mechanism by which LexA repressor increases the susceptibility of RecA protein to challenge by SSB protein was precluded because the effector concentration was decreasing with time due to cleavage. These limitations can be circumvented by using an uncleavable mutant LexA repressor, an approach that is detailed in the accompanying article (21). Direct measurement of RecA protein-ssDNA complex formation in the presence of uncleavable LexA repressor supports the conclusion that the repressor does not alter the equilibrium binding properties of the RecA protein for ssDNA but demonstrates that the repressor does reduce the rate at which the RecA protein displaces the SSB protein from ssDNA (21).

Reconstructions of electron micrographs demonstrate that LexA repressor protein interacts within the deep helical groove of the RecA nucleoprotein filament (20), overlapping a region of

RecA protein proposed to be a secondary DNA binding site (65). In support of this structural observation, the addition of dsDNA (Fig. 5) or the presence of excess ssDNA ((52, 66); Fig. 6*A*) inhibits the ability of the RecA protein-ATP-ssDNA complex to stimulate LexA repressor cleavage. Several lines of biochemical evidence indicate that the reduction in proteolytic cleavage activity is a direct consequence of the binding of a second DNA molecule by the RecA protein ternary complex and is not due to the nonspecific interaction of the LexA repressor with ds- or ssDNA. Just as for the cleavage of the lambda cI repressor (44), dsDNA decreases LexA proteolysis in a concentration-dependent manner. Suppression of LexA repressor cleavage by dsDNA is contingent on magnesium ion concentrations that support the DNA pairing activity of the RecA protein (Fig. 5), arguing for a need to bind dsDNA. Inhibition of LexA repressor cleavage resulting from excess ssDNA saturates at ratios of ssDNA to RecA protein that are known to be optimal for aggregation and renaturation of ssDNA (53, 54), while being stoichiometrically alleviated by the presence of the SSB protein (Fig. 6*B*). Moreover, the extent of inhibition of LexA repressor cleavage caused by various types of ssDNA correlates with the binding preference displayed by RecA protein (Fig. 6*A*). These results are most consistent with the interpretation that the RecA protein-ATP-ssDNA complex interacts with either a secondary DNA molecule or the LexA repressor in a mutually exclusive and, therefore, competitive manner.

Various *in vivo* observations support the idea that the binding of a specific set of target proteins and a secondary DNA molecule to the RecA nucleoprotein filament is mutually exclusive. The RecA protein maintains multiple roles in SOS mutagenesis (67). Initial cleavage of the LexA repressor inducing expression of the UmuC and UmuD proteins, which are essential to mutagenesis, is followed by RecA protein stimulating proteolytic maturation of the UmuD protein to yield a functional UmuD′ polypeptide. Finally, the RecA protein may function in targeting UmuD′C protein complexes to sites of DNA damage where DNA polymerase complexes are stalled. Interestingly, overexpression of UmuD′C proteins reduces genetic recombination *in vivo* by 50-fold in the absence of DNA damage (68); in addition to the reduction in recombination correlating with the amount of mutagenic complex, the fact that constitutive high concentrations of RecA protein partially alleviate this inhibition suggests that interaction of the UmuD′C complex with the RecA nucleoprotein filament is directly responsible for reduced levels of recombination (68). This view is further substantiated in the accompanying article where it is demonstrated that LexA repressor blocks the DNA strand exchange activity of the RecA protein *in vitro* (21).

Although the conclusion that the processes of SOS induction or mutagenesis may compete with recombination is mechanistically insightful, the biological significance of this competition would be suspect if the timing of these events did not coincide. During bacterial conjugation, DNA enters a recipient cell in a single-stranded form before being converted, at least in part, to duplex DNA by lagging strand synthesis. Several conjugal plasmids possess a gene, designated *psiB*, which permits both the transfer of ssDNA and its integration into the recipient chromosome by genetic recombination, without inducing the SOS response (69, 70). The psiB protein does not directly inhibit expression of SOS regulon genes (69) but prevents proteolytic inactivation of the LexA repressor (71) by altering either generation or processing of an SOS inducing signal. Furthermore, since its ability to impede SOS induction varies both as a function of the *recA* allele employed and the nature of the inducing treatment, the psiB protein may perturb a functional interaction between RecA and LexA proteins by binding to the RecA protein (71). While the molecular basis for psiB protein function is not yet fully understood, it clearly serves to prohibit induction of the SOS response without interfering with conjugal recombination (72) and, thus, may prevent competitive reactions from occurring simultaneously. Both the evolution of psiB function and the antagonistic effect of the UmuD′C protein complex on genetic recombination potentially support *in vitro* results indicating that binding of target proteins (*i.e.* LexA and lambda cI repressors) or a secondary DNA molecule by the RecA protein are mutually exclusive events. Consequently, the cellular processes of SOS induction/mutagenesis and genetic recombination may be intrinsically competitive.

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