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Mechanism of action of the *lexA* gene product

(multiple operator sites/symmetrical major groove interactions/regulation of SOS response/bacteriophage repressors)

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ABSTRACT **Genetic experiments have suggested that the *lexA* gene product of *Escherichia coli* represses a number of genes involved in the response to DNA damage, including *recA* and *lexA*. We purified the *lexA* gene product from bacterial strains that bear plasmids that direct the synthesis of large amounts of the protein. Purified *lexA* protein bound to two symmetrical DNA sequences in front of *lexA* and one in front of *recA*, protecting them from digestion with DNase I and blocking methylation of purines in the major groove. *lexA* protein repressed transcription of both genes *in vitro*. *lexA* protein binds to the two sites in front of the *lexA* gene with approximately the same affinity and with greater affinity to the single site in front of the *recA* gene. The affinity of *lexA* protein for its operator sites was measured under conditions that mimic conditions *in vivo*. Differences in the affinity with which *lexA* protein binds to the operators of genes it represses may account for the differences in the timing and extent of their induction after DNA damage.**

Various agents that damage DNA (e.g., UV light, activated carcinogens) induce an array of functions in *Escherichia coli* (1, 2). One step in this "SOS response," as it is called, is the activation of the *recA* protein, which then cleaves, and thereby inactivates, the *lexA* protein (reviewed in ref. 3). The latter is a repressor of *recA*, and its inactivation (4) results in increased synthesis of *recA* protein. A number of other genes are induced during the SOS response (refs. 5 and 6; unpublished). Genetic studies have suggested that there are two classes of these induced genes: 1, those that are repressed by *lexA* protein, and 2, those that are controlled by other *recA*-sensitive repressor proteins.

Initial experiments suggested that class 1 includes two genes, one involved in filamentation (7, 8), and the *lexA* gene itself (8, 9). Similar experiments have implied that *lexA* protein represses the *uvrA* gene, which is involved in DNA repair (10, 11); *sfiA*, which is necessary for the filamentous growth observed after DNA damage (12); *himA*, required for integration of λ and related bacteriophages (H. Miller, personal communication); *umuC*, the function of which is necessary, but not sufficient, for the mutagenic repair of DNA (A. Bagg, C. Kenyon, and G. Walker, personal communication); and certain colicins (13–15). Many other loci, so far defined only by insertions of phage Mu(Ap^r, *lac*) (5), appear to be repressed by *lexA* protein (C. Kenyon and G. Walker, personal communication), and it is possible that other *lexA*-repressible genes exist in *E. coli*.

Class 2 of induced genes is exemplified by the genes expressed during lytic growth of the lambdoid phages (refs. 16 and 17 and references therein). The cleavage of phage repressor proteins by *recA* protein, which leads to the induction of these genes after DNA damage, is well understood biochemically (18, 19). It is possible that some gene involved in the induced ability to repair DNA (other than *umuC*) is repressed by a *recA*-sensitive repressor protein other than the *lexA* protein (ref. 20; unpublished).

The genes involved in the SOS response manifest a spectrum of responses to the DNA damage that induces them: they differ in their sensitivity to inducing treatments, the amount of their induction, and the length of time it takes them to become induced. For example, transcription of the *sfiA* gene is induced quickly (12), whereas expression of the *dinD* locus is induced slowly (5), after treatment with mitomycin C. Ratios of induced to basal transcription vary from 4:1 for *lexA* (unpublished) to more than 30:1 for the *dinA* locus (5).

The experiments described herein demonstrate that purified *lexA* protein represses, *in vitro*, two of the genes induced after DNA damage. The mechanism of this repression is the binding of the protein to specific homologous sequences in front of the two genes. These sequences differ in their number, positioning relative to the promoter, and affinity for the *lexA* protein. It is probable that identical or nearly identical operator sequences, some with different affinities for the protein, will be found in front of other genes that *lexA* protein represses.

While this work was in progress, J. Little and D. Mount described to us transcription and DNase protection experiments with partially purified *lexA* protein that led to similar conclusions (21).

MATERIALS AND METHODS

Bacterial Strains. RB405 (W3110 *dam-3*) and MM294 (*end hsdR thi pro*) were provided by M. Meselson. Construction of RB791 (W3110 *lac*I^{q}L$_8$) will be described elsewhere.

Manipulation of Nucleic Acids *in Vitro*. Strand separation, 5′-end-labeling, and DNA sequence analysis were done as in ref. 22; 3′-end-labeling, with DNA polymerase I as in ref. 23. Techniques for constructing plasmids followed refs. 8 and 24. Exonuclease BAL 31, a gift of Jacqueline Miller, was used at 22°C in the buffer of Gray *et al.* (25).

Characterization of Control Regions. Plasmid pRB160 (8) was a source of *lexA* control region DNA. It was 3′-end-labeled at either the *Bcl* I site or the *Eco*RI site and digested with the other enzyme. The sequences of separated strands were determined as in ref. 22. The *Eco*RI/*Bcl* fragment was transcribed *in vitro* and the precise size of the runoff transcript was determined by comparison with a series of RNA molecules that differed in length by only a single nucleotide. For the experiments that measure repression of *lexA* transcription *in vitro*, pRB181, a plasmid that contains the *lexA* promoter fragment flanked by *Hin*dIII ends (unpublished), was used. pRY103, a plasmid that contains the *recA* control region near a *Bam*HI site (R. Yocum, personal communication), was cut with *Bam*HI, and the resulting ends were either 3′- or 5′-labeled, then cut with *Hae* III. DNase I protection ("footprinting") (26, 27), to determine the stretch of DNA covered by *lexA* protein, and the affinity of each site for the protein were studied as described by Johnson *et al.* (26). (DNA concentration was kept as low as practicable,

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about 0.1 nM. At this concentration, which is probably below the dissociation constant for *lexA* protein binding to its strongest site, the apparent K_d is approximately equal to the concentration of protein required to occupy one-half of the operator molecules. This concentration is determined by inspection of the autoradiogram. Because the proportion of *lexA* protein molecules at a given concentration of this preparation competent to bind DNA is not known, actual affinities could be tighter than those measured here, and only the relative affinities should be compared.) Dimethyl sulfate experiments to reveal purines covered by bound *lexA* protein (28) were done analogously to the DNase protection experiments except that each 200-μl methylation reaction was performed in 10 mM sodium cacodylate, pH 7.0, instead of Tris, and with 1 μl of a 2% solution of dimethyl sulfate in dioxane added instead of DNase. After the reaction had been halted and the DNA had been precipitated, the partially methylated fragment was resuspended in 100 μl of 20 mM ammonium acetate/0.1 mM EDTA, pH 8.0, at 90°C for 15 min, and piperidine was added to 1 M. The mixture was heated at 90°C for another 15 min then treated as described (22), and the products were separated on an 8% sequencing gel.

pRB192. Construction of pRB192, a plasmid that directs the synthesis of large amounts of *lexA* protein, will be described in detail elsewhere. Briefly, DNA in front of the *lexA* gene was resected with exonuclease BAL 31. A fragment of DNA bearing the *lac* UV5 promoter (ref. 29; G. Lauer, personal communication) was installed to transcribe the gene, strain MM294 was transformed with the ligation mixture, and cells that bore plasmids that directed the synthesis of large amounts of *lexA* protein were identified by their small colony size, their extraordinary sensitivity to ultraviolet radiation (8), and their ability to produce a large amount of a new protein that comigrated on Laemmli gels (30) with ^{35}S-labeled *lexA* protein. The *lac* promoter–*lexA* gene fusion that was most efficient at directing *lexA* protein synthesis was excised and inserted into the thermoinducible cloning vector pAS2 (unpublished) to create pRB192. After induction with heat and isopropyl β-D-galactopyranoside, *lexA* protein constituted 2–5% of the total protein of these cells (Fig. 2).

***lexA* Protein.** The *lexA* gene product was purified from lysates of RB791/pRB192 by precipitation with polyethyleneimine, followed by back extraction with KCl, concentration with ammonium sulfate, and chromatography on phosphocellulose, Bio-Gel P-150, and Affi-Gel 501 (both from Bio-Rad) (see Fig. 2). *lexA* protein passed through the organomercurial agarose column material Affi-Gel 501 while dozens of contaminating proteins were retained. This was anticipated because *lexA* protein contains no cysteine residues (14) and should be unable to interact with this column. The protein was assayed and purification was monitored by gel electrophoresis (30). Protein from the phosphocellulose column and from subsequent steps was active as judged by its ability to specifically repress transcription from the *recA* and *lexA* promoters *in vitro* (not shown).

RESULTS

***lexA* Control Region.** We determined the sequence of a *Bam/Bcl* fragment (8) that contained the *lexA* promoter. The sequence 5′ to the *lexA* gene revealed similarities to the *recA* control region (31, 32). The sequence contained two (or even three) sequences with approximate twofold rotational symmetry homologous to one such sequence in front of the *recA* gene (see Fig. 1). We speculated that these sequences in front of the two genes were sites to which *lexA* protein bound to repress their transcription. The startpoint of transcription (shown as +1 in Fig. 1) was deduced from the DNA sequence after measurement of the precise length of the runoff transcript on denaturing gels (see *Materials and Methods*). This transcriptional start is the same as that found by determining the sequence of the 5′ end of the *in vitro* transcript (14, 33).

***lexA* Protein.** We purified *lexA* protein from bacterial strains that bore plasmids that directed the synthesis of large quantities of the *lexA* protein (*Materials and Methods* and Fig. 2). On a Bio-Gel P-150 sizing column almost all of the *lexA* protein, which has an apparent monomer molecular weight of about 24,000 (8, 9), appeared dimeric at concentrations between 1 and 10 μM. At the end of the purification, only one contaminant was visible on overloaded $NaDodSO_4$ protein gels. This contaminant appears to form mixed oligomers with *lexA* protein and may be the carboxyl-terminal fragment of *lexA* protein, generated by *recA* proteolysis *in vivo* in the producing strain or during an early stage of the purification (unpublished).

Repression of Transcription *in Vitro*. *lexA* protein repressed, *in vitro*, transcription from the *lexA* promoter and from the *recA*

FIG. 1. Organization of the *lexA* and *recA* control regions. Sequence of DNA 5′ to the *recA* gene (30, 31) is shown, together with a portion of the sequence 5′ to the start of the *lexA* gene. Operator sites to which *lexA* protein binds to repress transcription of these two genes are boxed. Startpoint of *lexA* transcription was determined as described in the text. Regions of each strand protected by *lexA* protein from digestion with DNase I are indicated by brackets. Gs protected from methylation by dimethyl sulfate by bound *lexA* protein are circled; those whose methylation was enhanced by the bound protein are indicated with a caret. Protection of the G at −2 on the top strand of the *lexA* operator region is weak under the "physiological" conditions at which the experiment was performed. There is an uncertainty of approximately one base pair in assigning the boundaries of the regions protected from DNase. Two bases within the DNase protected region in front of *lexA* showed enhanced cleavage even in the presence of ≥15 nM *lexA* protein. These occurred at −2 and at +17 in the bottom strand. A third area of weaker homology, centered between −37 and −38, was not protected by *lexA* protein in any experiments (not shown).

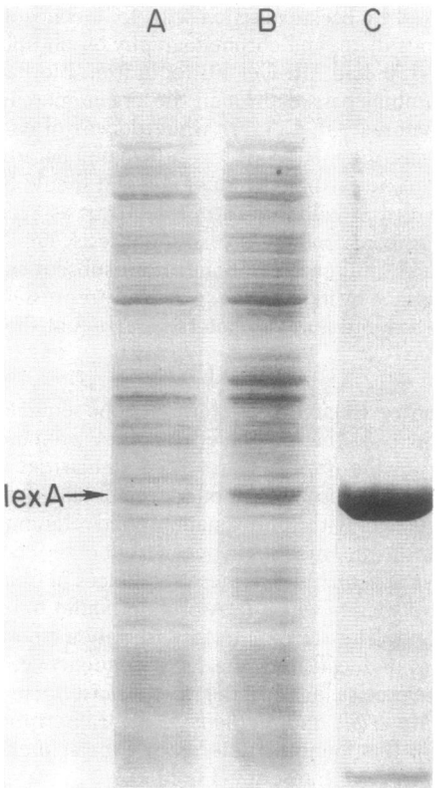

FIG. 2. *lexA* protein in RB791/pRB192 grown at 30°C (lane A), after growth at 40°C for 4 hr with isopropyl β-D-galactopyranoside added to 0.1 mM 1 hr after temperature shift-up (lane B), and after purification (lane C). *lexA* protein was purified from 70 g of frozen RB791/pRB192. The frozen cell mass was suspended in 150 ml of 10 mM EDTA/1 mM dithioerythritol/5% (vol/vol) glycerol/5 mM NaCl/ 23 μM phenylmethylsulfonyl fluoride at 0°C and lysed by sonication (34). Tris base was added dropwise during the sonication to keep the pH above 6.5. After lysis was >90% complete as judged by OD_{550}, the sonicated cell extract was centrifuged at 4°C for 40 min at 16,000 × *g*. All subsequent manipulations were done at 4°C. The standard buffer used throughout the preparation contained 50 mM Tris·HCl at pH 7.4, 1 mM EDTA, 1 mM dithioerythritol, 5% glycerol, and salt as indicated. The supernatant was diluted 1:3 with standard buffer and made 2% in polyethyleneimine, and the resulting suspension was centrifuged for 40 min at 16,000 × *g*. The polyethyleneimine pellet was resuspended in standard buffer plus 200 mM KCl and centrifuged again at 16,000 × *g* for 40 min, $(NH_4)_2SO_4$ was added to the supernatant with gentle stirring to 70% (wt/vol), and the suspension was centrifuged at 16,000 × *g* for 40 min. The pellet from this centrifugation was dissolved in standard buffer plus 200 mM KCl, dialyzed twice for 90 min each against standard buffer with 75 mM KCl and 70 mM potassium phosphate, pH 7.0, instead of Tris, loaded directly onto a 20 × 0.5 cm phosphocellulose column, and eluted with a linear gradient of 0.1–1.5 M KCl. *lexA* protein eluted from the column in the first third of the gradient, and peak fractions were concentrated with ammonium sulfate and back extracted as above, dialyzed against standard buffer plus 200 mM NaCl, and loaded onto a 1 m × 1 cm column of Bio-Gel P-150, 100–200 mesh. *lexA* protein eluted from the column at a position expected for a globular protein with a molecular weight approximately twice that of a *lexA* monomer. Peak column fractions that contained *lexA* protein were pooled, dialyzed against standard buffer plus 200 mM NaCl without dithioerythritol, and allowed to drip through a 5-ml organomercurial agarose column (Affi-Gel 501). The organomercurial agarose flowthrough was made 1 mM in dithioerythritol, divided into aliquots, and stored at −20°C.

promoter, but not from the β-lactamase promoter of pBR322 (Fig. 3). Under these conditions, transcription from the *recA* promoter appeared to be repressed more at lower concentrations of *lexA* protein than was transcription from the *lexA* promoter.

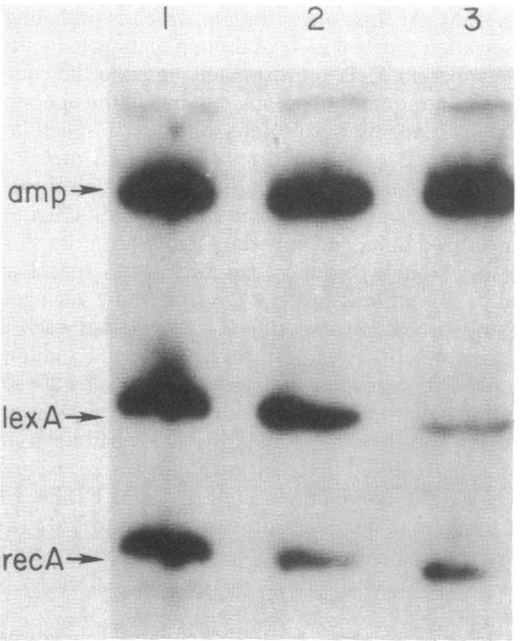

FIG. 3. Repression of transcription from the *lexA* and *recA* promoters *in vitro*. Reactions took place in 20-μl mixtures essentially as described in ref. 35. Mixtures contained, at approximately 4 nM each, a 450-base pair *Eco*RI/*Hin*dII fragment from pBR322 that contained the β-lactamase promoter, a 250-base pair *Hin*dIII/*Hin*dIII fragment from pRB181 that contained the *lexA* promoter, and an approximately 280-base pair *Hae* III/*Bam* fragment from pRY103 that contained the *recA* promoter. After a 15-min incubation with *lexA* protein, RNA polymerase (New England BioLabs) was added to about 100 nM. After 10 min, nucleotides and heparin were added to the reaction mixture in the following concentrations: heparin at 100 μg/ml, ATP at 250 μM, CTP and GTP at 50 μM each, and [α-^{32}P]UTP at 22.5 μM. Lane 1, no *lexA* protein added; lane 2, *lexA* protein added to 20 nM; lane 3, *lexA* protein added to 200 nM. β-Lactamase is indicated by *amp*.

Delineation of *lexA* Protein Binding Sites. Purified *lexA* protein protected sites in the control regions of *recA* and *lexA* from digestion with DNase I (see Fig. 4). In the *recA* control region, *lexA* protein protected a region that included the proposed binding site and extending for 5–6 nucleotides 3′ and 2–3 bases 5′ on both strands. In the *lexA* control region, a single large protected area included both boxed regions and a stretch of flanking DNA similar to that found for the single site in front of *recA* (Fig. 1). The bottom strand of the *lexA* control region contains within the protected area two bases at which DNase cleavage is enhanced by bound *lexA* protein at concentrations ≥15 nM (legend to Fig. 1). The most prominent of these enhanced DNase I cleavages is near the space between the two binding sites.

To better localize bases involved in binding by *lexA* protein, we examined the ability of bound *lexA* protein to block methylation of purines by dimethyl sulfate. Bound proteins can block the methylation of the N7 of guanines and the N3 of adenines, atoms that lie, respectively, in the major and minor grooves of B form DNA (28). *lexA* protein protected some Gs in its operator sites from methylation with dimethyl sulfate and enhanced the reactivity of others (see Fig. 1). Gs at identical positions in the three sites, two in the bottom strand and one in the top strand, were protected from methylation. Gs protected by bound *lexA* protein in the sites are near the periphery of the 20-base pair sequence, whereas the reactivity of the Gs closer to the core of the sequence tended to be either unaffected or enhanced. *lexA* protein did not affect methylation of any As in either control region, suggesting that, as is the case for other repressor

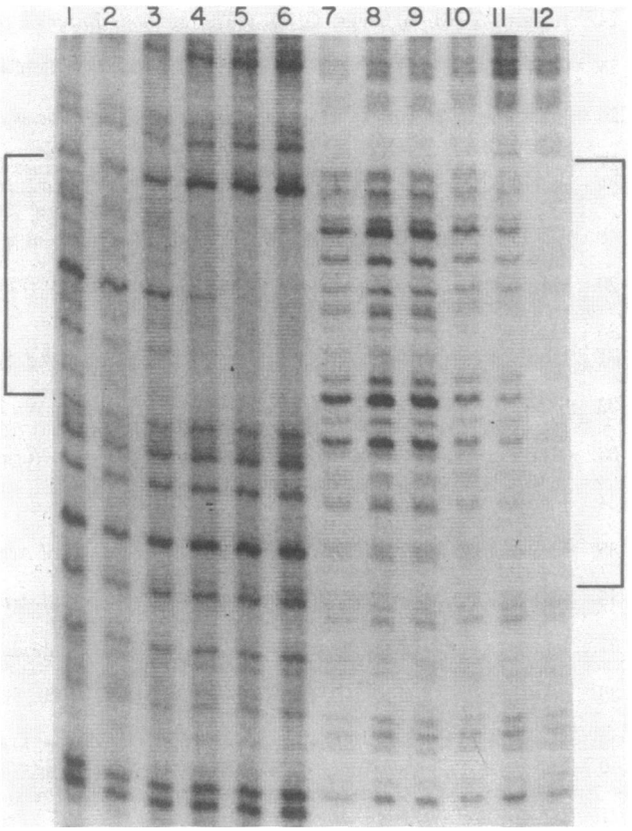

FIG. 4. Protection of the *recA* and *lexA* control regions from digestion with DNase I. Lanes 1–6, *recA*: the *Bam/Hae* III fragment from pRY103, 3′ labeled at the *Bam* site; lanes 7–12, *lexA*: the *Eco*RI/*Bcl* fragment from pRB160, 3′ labeled at the *Bcl* site. Both fragments were treated with DNase I. *lexA* protein concentrations were: lane 1, 0 nM; lane 2, 0.5 nM; lane 3, 1.5 nM; lane 4; 5 nM; lane 5, 15 nM; lane 6, 50 nM; lane 7, 0 nM; lane 8, 1.5 nM; lane 9, 5 nM; lane 10, 15 nM; lane 11, 50 nM; lane 12, 150 nM. Brackets indicate portions of the control regions that were protected by *lexA* protein from digestion with DNase I.

proteins (refs. 36, 37, and 38 and references therein), the purine contacts important for *lexA* protein binding are mainly in the major groove. Significantly, no G was protected outside the boxes shown in Fig. 1.

The above data show that these sequences constitute operator sites for *lexA* protein. The fact that *lexA* protein binding sites overlap stretches of both sequences that contain important points of contact with the RNA polymerase in promoters that have been well studied (38) suggests that the mechanism by which it blocks transcription of these two promoters is probably exclusion of RNA polymerase, and other experiments suggest that this is true; *lexA* protein added before (but not after) RNA polymerase represses transcription of both promoters (not shown).

Binding Affinities. Equilibrium dissociation constants for *lexA* protein binding to the *recA* and *lexA* control regions were measured as that concentration of the protein that resulted in half-maximal protection of the operator sites. All protection experiments described in this work were performed under conditions (37°C, 200 mM KCl) similar to those found *in vivo* (26). At equilibrium it takes about 10 times more *lexA* protein to half-maximally fill the two sites in front of the *lexA* gene than it takes to half-maximally fill the site in front of *recA* (see Fig. 3). These experiments allow us to place maximal values on the K_d of *lexA* protein for the *recA* and *lexA* operators under these conditions—about 2 nM and 20 nM, respectively. Because the fraction of protein competent to bind operator at these concentrations is not yet known, the actual interaction may be tighter. Although they differ substantially in sequence, the two sites in front of the *lexA* gene are occupied at or nearly at the same concentration of *lexA* protein. Preliminary experiments (unpublished) indicate that there is an interaction (26) between *lexA* protein molecules bound to these adjacent sites.

DISCUSSION

These experiments have demonstrated that *lexA* protein binds to operator regions of similar sequence in front of two genes it represses. It is likely that one or more of these sequences, or variants of them, will be found in front of other *lexA*-repressed genes. The *lexA* protein binds to two sites in front of its own gene but only to one in front of the *recA* gene; we do not yet understand the reason for this difference.

The results of the equilibrium dimethyl sulfate and DNase I protection experiments are consistent with the idea that the interaction of *lexA* protein with its operators resembles that found for the bacteriophage repressors: the dimethyl sulfate experiments argue that the protein interacts primarily with the major groove, rather than the minor groove, and both sorts of experiments argue that the interaction is symmetrical. The site to which *lexA* protein binds most tightly shows almost perfect twofold rotational symmetry, while two sites to which it binds more weakly deviate considerably from perfect symmetry.

The *lexA* protein binding sites are located differently with respect to the two promoters (Fig. 1). Compared with the site in the *recA* control region, the two sites in front of the *lexA* gene are displaced roughly one and three turns of the helix farther from the promoter's −35 region, toward the structural gene. It is possible that an equivalent amount of occupancy of these binding sites in different regions of the two promoters might produce different amounts of repression of transcription. Binding sites for other regulatory proteins that act at many sites in the cells genome, such as cyclic AMP-binding protein (39–41) and *trp* repressor (42), are also found at different positions relative to the promoter.

The observed difference in affinity of *lexA* protein for two different control regions allows at least a partial explanation for the timing of induction of SOS functions. As the level of *lexA* protein within a cell begins to decrease after DNA damage has occurred, due to cleavage by *recA* protein, the *lexA* promoter should become derepressed before the *recA* promoter becomes proportionately derepressed. Due to the self-repression of *lexA* protein synthesis (8, 9), inactivation of lexA protein results in increased transcription from the *lexA* promoter (unpublished). The increase in *lexA* protein synthesis will tend to prolong the period during which the level of *lexA* protein is dropping. Other *lexA*-repressed operators might bind the protein with affinities intermediate between those of the *lexA* and *recA* control regions; these would become effectively derepressed before the *recA* promoter is efficiently induced, perhaps allowing the cell to initiate DNA repair to remove the damage and halt the fall in *lexA* protein levels before large amounts of *recA* protein are synthesized and prophages are induced.

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