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 A number of other genes are induced during the SOS response (refs. 5 and 6; unpublished).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); sfa, which is necessary for the filamentous growth observed after DNA damage (12); hnaA, required for integration of A 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', 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 sfa gene is induced quickly (12), whereas expression of the dinD locus is induced slowly (3), 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 hasr thi pro) were provided by M. Meselson. Construction of RB791 (W3110 lacFPla) 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 EcoRI site and digested with the other enzyme. The sequences of separated strands were determined as in ref. 22. The EcoRI/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 HindIII ends (unpublished), was used. pRY103, a plasmid that contains the recA control region near a BamHI site (R. Yocom, personal communication), was cut with BamHI, 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, so that the white bands are not as intense as the black bands (28).)
about 0.1 nM. At this concentration, which is probably below the dissociation constant for *lexA* protein binding to its strongest site, the apparent *Kₐ* 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 *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 methyalted 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 pipetted 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 35S-labeled *lexA* protein. The *lac* promoter-*lexA* gene fusion that was most efficient at directing *lexA* protein synthesis was excised and inserted into the thermostable 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 NaDodSO₄ 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* promoter. In the absence of a promoter, this activity was seen at high levels of protein. The repression of *lexA* transcription was found to be a function of the amount of *lexA* protein present, with the most efficient repression occurring at a concentration of 10 μM *lexA* protein. The repression was specific, in that no repression of *recA* transcription was seen at any concentration of *lexA* protein tested.

**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 +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).
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. 2. lexA protein in RB791/pRB192 grown at 30°C (lane A), after growth at 40°C for 6 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 (54). Tris base was added dropwise during the sonication to keep the pH above 6.5. After lysis was >90% complete as judged by OD600, 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, (NH₄)₂SO₄ 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 × 1 cm column of Bio-Gel P-150. lexA protein 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 organomericulagarse column (Affi-Gel 501). The organomericulagarse flowthrough was made 1 mM in dithioerythritol, divided into aliquots, and stored at -20°C.

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 mM each, a 450-base pair EcoRI/HindIII fragment from pBR322 that contained the β-lactamase promoter, a 250-base pair HindIII/HindIII fragment from pBR181 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-32P]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
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 $K_d$ for 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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