Isolation and Characterization of Noncleavable (Ind⁻) Mutants of the LexA Repressor of *Escherichia coli* K-12

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The LexA repressor of *Escherichia coli* represses a set of genes that are expressed in the response to DNA damage. After inducing treatments, the repressor is inactivated in vivo by a specific cleavage reaction which requires an activated form of RecA protein. In vitro, specific cleavage requires activated RecA at neutral pH and proceeds spontaneously at alkaline pH. We have isolated and characterized a set of *lexA* mutants that are deficient in in vivo RecA-mediated cleavage but retain significant repressor function. Forty-six independent mutants, generated by hydroxylamine and formic acid mutagenesis, were isolated by a screen involving the use of operon fusions. DNA sequence analysis identified 20 different mutations. In a *recA* mutant, all but four of the mutant proteins functioned as repressor as well as wild-type LexA. In a strain carrying a constitutively active *recA* allele, *recA730*, all the mutant proteins repressed a *sulA::lacZ* fusion more efficiently than the wild-type repressor, presumably because they were cleaved poorly or not at all by the activated RecA protein. These 20 mutations resulted in amino acid substitutions in 12 positions, most of which are conserved between LexA and four other cleavable proteins. All the mutations were located in the hinge region or C-terminal domain of the protein, portions of LexA previously implicated in the specific cleavage reactions. Furthermore, these mutations were clustered in three regions, around the cleavage site (Ala-84–Gly-85) and in blocks of conserved amino acids around two residues, Ser-119 and Lys-156, which are believed essential for the cleavage reactions. These three regions of the protein thus appear to play important roles in the cleavage reaction.

In normal cell growth, the LexA repressor of *Escherichia coli* represses a set of genes called the SOS regulon (29, 49). When DNA is damaged by treatments such as UV irradiation or mitomycin C, another regulatory protein, RecA, is activated by an inducing signal, probably single-stranded regions of damaged DNA. The activated RecA protein in turn inactivates LexA repressor through a specific proteolytic cleavage near the center of the repressor. The cleavage of LexA repressor then leads to the derepression of the SOS genes and turns on the SOS response. If the cell contains a λ prophage, the activated RecA can also inactivate λ cI repressor by a similar cleavage event at the same peptide bond (alanine-glycine) as that cleaved in LexA (16), leading to prophage induction.

It is known that RecA protein is required for repressor cleavage in vivo, since a *recA* mutation blocks SOS induction and prophage induction (51). Activated RecA is also required in vitro under physiological conditions for repressor cleavage (6, 41, 42, 44). However, at high pH LexA repressor can break down spontaneously in the absence of RecA (25). This autodigestion reaction cleaves the same alanine 84-glycine 85 bond as is cut in the RecA-mediated cleavage reaction. λ cI repressor can also break down spontaneously under similar conditions to yield products identical to those resulting from the RecA-mediated cleavage; however, autodigestion of λ repressor proceeds at a much slower rate than does that of LexA repressor, a difference similar to that observed in RecA-mediated cleavage.

These findings indicate that LexA and λ cI repressors can carry out the specific cleavage by themselves under certain conditions. The same cleavage site specificity and substrate specificity in autodigestion and RecA-mediated cleavage suggest that these two types of cleavage reactions may

follow, at least in part, a common pathway. We have proposed (25, 47, 48) that, in the RecA-mediated cleavage reaction, the catalytic activity for the cleavage of LexA and λ cI repressor resides in the repressor itself, rather than in RecA protein, and that, under physiological conditions, activated RecA acts as a positive effector to stimulate the autodigestion reaction, rather than itself being a protease (14, 25, 47, 48). According to this model, cleavage involves several conceptually separable sites: (i) the Ala-84–Gly-85 cleavage site, which could be considered as the substrate for the reaction; (ii) the active site for the chemistry of the cleavage reaction, which may be considered to include both the groups that interact with the cleavage site and those which carry out the chemistry of bond rearrangement; (iii) the binding site for RecA; and (iv) the portion of the protein that transduces or couples the binding of RecA to the stimulation of autodigestion.

To help define the structural determinants in LexA repressor which are required for RecA-mediated cleavage, we have isolated a set of Ind⁻ mutants which prevent RecA-mediated cleavage in vivo. In this paper, we report the isolation and sequence analysis of 46 independent Ind⁻ mutants, their mitomycin C sensitivity, and their repressor function. These data help us to define regions of the LexA repressor important for cleavage and support previous conclusions that particular residues within these regions play important roles in the chemistry of cleavage. A parallel set of studies has been carried out with λ repressor by Gimble and Sauer (13, 14).

MATERIALS AND METHODS

Enzymes and chemicals. Restriction endonucleases were from New England Biolabs, Bethesda Research Laboratories, and Boehringer Mannheim; Moloney murine leukemia virus reverse transcriptase was from Bethesda Research

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TABLE 1. Bacterial, plasmid, and phage strains

Strain	Relevant markers	Source or reference
Bacterial strains		
AB1157	<i>recA</i> ⁺ <i>srl</i> ⁺ <i>lexA</i> ⁺	24
DE192	$\Delta(lac-gpt)5$ <i>recA</i> ⁺ <i>lexA51</i> <i>sulA211</i>	10
GC3217	<i>recA441</i> <i>sulA211</i>	12
JL623	$\Delta(lac-pro)$ <i>lexA</i> ⁺ <i>recA</i> ⁺ /F' <i>lacI</i> ^q 72 (=DM1884)	D. Mount
JL795	$\Delta(lacIPOZYA)169$ <i>sup</i> ⁺ <i>sulA211</i> <i>recA</i> ⁺ <i>lexA</i> ⁺ <i>rpsL31</i> <i>pro</i> ⁺ (=GE2265)/F' <i>lacI</i> ^q	28
	<i>lacZ</i> $\Delta M15::Tn3$	
JL852 ^a	<i>recA730 srl-300::Tn10 sulA211 lexA71::Tn5 rpsL31</i>	D. Ennis
JL855 ^b	JL852/F' <i>lacI</i> ^q 72	This work
JL859 ^c	JL855 (λ <i>recA::lacZ</i> <i>cI</i> <i>ind</i> <i>att</i> ⁺)	This work
JL932 ^d	<i>recA</i> ⁺ <i>srl</i> ⁺ derivative of JL859	This work
JL1047 ^d	<i>recA</i> ⁺ <i>srl</i> ⁺ derivative of JL1420	This work
JL1158	YMC(pMC9)	This work
JL1159	YMC(pJWL42)	This work
JL1246 ^e	$\Delta(recA-srlR)306::Tn10$ derivative of JL932	This work
JL1420 ^f	JL855 (λ <i>sulA::lacZ</i> <i>cI</i> <i>ind</i> <i>att</i> ⁺)	This work
JL1752 ^e	$\Delta(recA-srlR)306::Tn10$ derivative of JL1047	This work
YMC	<i>supF</i> (=N720)	27
Plasmids		
pBR322	<i>bla</i> <i>tet</i>	3
pJWL42	pBR322 derivative carrying <i>lexA</i> ⁺ gene	31
pJWL59	pBR322 derivative carrying <i>tacP::lexA</i> ⁺ fusion	48
pJWL147	pBR322 derivative carrying <i>lacP::lexA</i> ⁺ fusion (a <i>SacI</i> silent site in <i>lexA</i> ⁺)	47
pJWL184	<i>lexA291</i> (containing <i>Bam</i> HI, <i>Sal</i> I, <i>Bst</i> EII, and <i>Bgl</i> II silent sites in the <i>lexA</i> ⁺ gene) version of pJWL147	This work
pMC9	Derivative of pBR322 carrying <i>lacI</i> gene	4
Phage		
M13mp8-42	M13mp8 derivative carrying <i>lexA</i> gene from pJWL42	This work
M13mp9-147	M13mp9 derivative carrying <i>lexA</i> gene from pJWL147	This work

^a =JM30-SP, derived by Evelyn Witkin from GC3217 (12, 23) by successive transductions of *recA730* linked to *srlC300::Tn10* and of the defective *lexA* allele *lexA71::Tn5* (20).

^b JL852 was mated with JL623 (=DM1884), obtained from M. Pfahl via D. Mount, followed by selection for streptomycin resistance and *pro*⁺ on M9-6aa plates.

^c JL855 was lysogenized with λ *recA::lacZ att*⁺ *cI* *ind* (operon fusion) (28).

^d JL859 (for JL932) and JL1420 (for JL1047) were each transduced with P1 *vir* grown on the *srl*⁺ *recA*⁺ strain AB1157, selecting for *Srl*⁺ transductants and screening for *RecA*⁺ phenotype by testing for formation of turbid plaques by λ *imm*⁴³⁴ *cI*⁺.

^e JL932 (for JL1246) and JL1047 (for JL1752) were each transduced with P1 *vir* grown on strain NO57 [$\Delta(recA-srlR)306::Tn10$]. Transductants were selected with tetracycline and then tested for deletion of *recA* by UV sensitivity.

^f JL855 was lysogenized with λ *sulA::lacZ att*⁺ *cI* *ind*.

Laboratories, and T4 DNA ligase was from New England Biolabs; [α -³²P]dATP (3,000 Ci/mmol) was purchased from Du Pont; hydroxylamine, formic acid, and hydrazine were purchased from Sigma Chemical Co.

Media. Minimal sorbitol plates contained M9 minimal medium (33) and 2% Difco agar, with 0.2% sorbitol as a carbon source and 0.03% arginine, 0.01% methionine, and 0.015% each isoleucine, valine, threonine, histidine, and leucine. M9-6aa plates contained the same components, except that sorbitol and methionine were omitted and 0.2% glucose was added. λ plates contained 1.5% Difco agar, 1% tryptone (Difco), and 0.5% NaCl. MacConkey plates contained 5% MacConkey-lactose agar (Difco). L broth contained 1% tryptone, 0.5% yeast extract (Difco), 1% NaCl, and 1 mM NaOH. When appropriate, tetracycline, ampicillin, or kanamycin was added at 10, 100, or 50 μ g/ml, respectively.

Bacterial strains and phages. The *Escherichia coli* strains, plasmids, and phages used in this study are listed in Table 1.

Isolation of λ *sulA::lacZ cI* *ind att*⁺ was performed as follows. A λ *att*⁺ *int* phage carrying a *sulA::lacZ* operon fusion and isolated by R. d'Ari from a Mu dI(Ap *lac*) (17, 18) insert into *sulA* was obtained from K. Peterson (Fig. 1). The promoter was cloned into λ gt11 (52) as shown; ligations were packaged in vitro (9), using packaging mixes derived from strains BHB2688 and BHB2690 (15). Phages were plated in the presence of 5-bromo-4-chloro-3-indolyl- β -D-galactopyra-

FIG. 1. Construction of the λ *sulA::lacZ att*⁺ *int*⁺ *cI857* *Sam100* phage. Boxed regions indicate the substituted region in the phage, and dashed regions indicate uncertainty as to its extent. The upstream *SacI* site is presumably in the bacterial DNA. The arms of the phages are not exactly to scale. Abbreviations: S, *SacI*; RI, *EcoRI*; H3, *HindIII*; B, *Bam*HI; X, *Xba*I; K, *Kpn*I.

noside on strain JL1158 at 37°C. Whereas λ gt11 formed colorless clear plaques and the parental *att* *sulA*::*lacZ* phage formed medium-blue turbid plaques, a small proportion of the plaques from the packaged phage were deep blue and clear, and two isolates were further characterized. Both isolates formed very pale blue, clear plaques on JL1159; they did not plate on *sup*⁺ strains, indicating that the right arm was derived from λ gt11. The left arm of the phage was also derived from λ gt11, as shown by restriction enzyme mapping (not shown) of the one isolate tested with *Eco*RI and *Kpn*I. Moreover, like λ gt11 but unlike the *att* fusion phage, they did not carry *lacY*, as shown by the inability to ferment melibiose at 42°C (2) of DE192 lysogens carrying *cl ind* derivatives of the recombinant phage (see below). They formed lysogens at 32°C which were inducible by high temperature. Each isolate was crossed with *λcl ind-1*, and the progeny were plated on JL795 in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Turbid blue plaques were isolated and characterized; the behavior of lysogens of these phage (see results) showed that *lacZ* was under LexA control.

Plasmids. Plasmids used in this work are listed in Table 1. pJWL59 is an overproducing derivative of pBR322 carrying a fusion of the *lexA* gene to the *tac* promoter (48). pJWL147 is a derivative of pJWL59, but contains a fusion of the *lexA* gene to the *lacUV5* promoter (31) instead of the *tac* promoter. It carries a silent change at amino acid 101, creating a *Sal*I site (47), and also carries the M13 origin of replication derived from pZ150 (54). pJWL184 is essentially the same as pJWL147, except that, in addition to the *Sal*I site, it also carries three silent changes at residues Ile-66, Glu-153, and Val-179/Asp-180 (see Fig. 2), creating sites for *Bam*HI, *Bst*EII, and *Bgl*II, respectively. These three sites were introduced individually by site-directed mutagenesis using uracil-containing single-stranded DNA (21) from M13mp8-42 (see below) as a template. An *Mlu*I-to-*Sal*I fragment of the *lexA* gene containing the *Bam*HI site was cloned into the backbone of pJWL147, yielding pJWL182. Fragments containing *Bst*EII and *Bgl*II sites were then cloned into pJWL182, followed by a phenotypic screen for LexA function in strain JL1246. M13mp8-42 and M13mp9-147 contain the *lexA* gene in an *Eco*RI-to-*Hind*III fragment from pJWL42 (31) or from pJWL147, cloned into M13mp8 or M13mp9, respectively.

Mutagenesis of plasmid DNA. *lexA*(Ind⁻) mutants were isolated on plasmids. Both naturally occurring and artificially engineered restriction sites near the hinge and carboxy domain (Fig. 2) were used to generate restriction fragments for localized random mutagenesis in the region of interest. For example, a fragment generated by cutting pJWL184 with *Sal*I and *Sna*BI, comprising amino acid residues 101 to 140 of the LexA protein, was mutagenized and then cloned into a wild-type plasmid backbone.

Two methods were used to mutagenize DNA: hydroxylamine treatment of double-stranded DNA, and formic acid or hydrazine treatment of single-stranded DNA. Hydroxylamine mutagenesis was performed using standard procedures (9). Formic acid or hydrazine mutagenesis was performed as described (37) with some modifications. A 50-μg sample of mp8-42 or mp9-147 single-stranded DNA was treated with 1.5 volumes of concentrated formic acid or hydrazine at room temperature for 5 min. The reaction was stopped by adding 2 volumes of 2.5 M sodium acetate and 20 μg of tRNA, followed by ethanol precipitation three times. A 40-pmol sample of oligonucleotide primer was annealed to treated DNA in 60 mM Tris hydrochloride (pH 8.3)–50 mM

FIG. 2. Unique restriction sites in the plasmid (pJL184) *lexA* gene. Sites in bold type are the silent sites artificially engineered into the *lexA* gene. a.a., Amino acid; the numbers indicate the position of the corresponding amino acid in the LexA protein sequence.

KCl–10 mM MgCl₂–2 mM dithiothreitol by treating the mixture at 75°C for 5 min and then at 40°C for 15 min. Then dATP, dCTP, dGTP, and TTP were added to a final concentration of 0.5 mM each. The reaction was incubated at 40°C for 1 h after the addition of 1,000 U of Moloney murine leukemia virus reverse transcriptase, followed by phenol extraction and ethanol precipitation. This DNA was digested with restriction enzymes to excise the desired fragment in double-stranded form, and this fragment was then gel purified and reinserted into an untreated plasmid vector for the mutant screen.

In hydroxylamine mutagenesis, localized mutagenesis was performed within the *Mlu*I-to-*Sal*I, *Sal*I-to-*Sna*BI, *Sna*BI-to-*Hind*III, *Eco*RV-to-*Bst*EII, *Bst*EII-to-*Bgl*II, and *Bgl*II-to-*Hind*III intervals in plasmid pJWL147 or pJWL184 (see Fig. 2). In addition to localized mutagenesis, intact plasmid DNA was also treated with hydroxylamine and used directly for mutant screening. In most experiments, including treatment of the intact plasmid, the proportion of white colonies among JL1420 transformants (see below) was 0.1 to 0.3%, except that within the intervals *Eco*RV to *Bst*EII, *Bst*EII to *Bgl*II, or *Bgl*II to *Hind*III, no white colonies were found among over 2,000 transformants. By comparison, the frequency of *lexA*(Def) mutants was 1 to 3% when the intact plasmid was treated with hydroxylamine, as judged by the frequency of formation of LacZ⁺ colonies in strain JL1752, a *recA* mutant derivative of strain JL1420 in which the *lexA*⁺ plasmid allows formation of a white colony.

In formic acid or hydrazine mutagenesis, single-stranded DNA of either mp8-42 or mp9-147 was used to mutagenize both complementary strands of the *lexA* gene. The mutagenesis was localized within *Mlu*I-to-*Sal*I, *Sal*I-to-*Sna*BI, and *Sna*BI-to-*Hind*III intervals. In most cases, the frequency of potential *lexA*(Ind⁻) mutants was 0.3 to 1%. However, when formic acid mutagenesis, using mp9-147 as a template, was localized within the *Sal*I-to-*Hind*III interval, no Ind⁻ mutants were found among 3,000 colonies.

Plasmids from potential mutants were isolated and retransformed into strain JL1420 to test whether the Lac⁻ phenotype was due to a change in the plasmid or in the indicator strain. In greater than 80% of the isolates, the mutation for the phenotype mapped to the plasmid. For such isolates, we then subcloned the fragment that had been mutagenized back into a wild-type backbone. This in vitro backcross served to map the mutation to the mutagenized fragment, to purify it from any potential adventitious mutations elsewhere in the gene, and to limit the extent of the gene which needed to be sequenced.

In all, 46 independent mutants, including four spontaneously occurring ones, were isolated and subjected to DNA sequencing. Among the 42 induced mutations, all but 3 were found to lie in the target region that had been mutagenized. Of the 46 spontaneous and induced mutations, 44 contained a single amino acid substitution; two isolates, KN156/QH184 and KT156/EV170, contained two amino acid changes each. By use of restriction enzymes and subcloning, each single

mutation in these two isolates was separated from the other. The phenotype in strain JL1420 showed that KN156 or KT156, but not QH184, was responsible for the full *Ind*⁻ phenotype. The EV170 mutation appeared to be a leaky *Ind*⁻ mutation. Plasmids bearing this single mutation in strain JL1420 formed pink colonies, whereas most of the *Ind*⁻ mutants formed white colonies.

Three spontaneously arising mutations, EK95, SL119, and a deletion of Ala-83 (AΔ83), were originally isolated in pJWL59, using strain JL859 as an indicator (see next section). When these mutant genes were placed under the control of the weaker *lacUV5* promoter, AΔ83 and SL119, but not EK95, still produced the *Ind*⁻ phenotype in JL1420. Therefore, the *Ind*⁻ phenotype of the EK95 mutation seems to exhibit a stronger dose dependence on the level of LexA repressor in the indicator strain than that observed with other mutations. Overall, excluding EK95 and EV170, which were not studied further, our mutant collection contains 20 different mutations resulting in amino acid substitutions in 12 positions.

Isolation of *lexA*(*Ind*⁻) mutants. The rationale for isolation of mutants is described in Results. In our initial experiments, we used the plasmid pJWL59, carrying a *tacP::lexA* fusion, and the indicator strain JL859 (carrying a *recA::lacZ* operon fusion). Three mutants were isolated with this system (see Results). However, two observations led us to switch from pJWL59 to pJWL147 (*lacP::lexA*⁺) and to change the indicator strain from JL859 to JL1420 (carrying a *sulA::lacZ* fusion). First, when the expression of LexA repressor was under the control of the strong *tac* promoter, as in pJWL59 derivatives, cells bearing even the uninduced level of a noncleavable repressor grew slowly and tended to accumulate plasmids with a nonfunctional *lexA* gene, presumably because these had a selective advantage even in cells carrying a mixed population of plasmids with functional and nonfunctional *lexA* genes. Therefore, a switch from the *tac* promoter to a weaker promoter, *lacUV5*, reduced the basal expression of the *lexA* gene and allowed normal growth rates. Second, with this weaker promoter, strain JL859 carrying the *lacP::lexA252*(*Ind*⁻) plasmid made a fairly red colony on a MacConkey plate. Presumably, the level of LexA252 repressor driven by the *lacUV5* promoter was too low to repress the *recA::lacZ* fusion. We therefore used an operon fusion to the *sulA* promoter, which has a much lower level of repressed expression than *recA* (18, 40). This fusion, which also lacks *lacY* (unlike the *recA::lacZY* fusion), gave a white colony with the *lacP::lexA*(*Ind*⁻) plasmid and a red colony with a white border with the *lacP::lexA*⁺ plasmid.

DNA sequence determination. The position of mutations was determined by direct plasmid sequencing using the dideoxy chain termination method (43) and Moloney murine leukemia virus reverse transcriptase to synthesize DNA (53). A set of primers that hybridized within or near the *lexA* gene was also used for the sequencing (although *oriM13* was present on most of our plasmids, it did not function well enough in this particular construction to give adequate amounts of single-stranded DNA for sequencing). For each mutation, sequence determination spanned only the fragment to which the mutation had been mapped (see above).

Mitomycin C sensitivity of mutants. Strain JL1420, carrying the wild-type *lexA* gene or a *lexA*(*Ind*⁻) mutant gene on pJWL147, was used to measure the sensitivity to mitomycin C treatment conferred by the wild-type or mutant repressor. Cultures were grown in exponential phase in L broth at 37°C and diluted 100-fold into 5 ml of saline (0.85% NaCl), and mitomycin C was added to a final concentration of 1 μg/ml.

A sample was immediately diluted 100-fold into ice-cold L broth for a zero-time control, and the remainder was incubated at 37°C for various times, followed by dilution as above. Portions of each dilution were then spread onto λ agar plates containing 100 μg of ampicillin per ml, which were then incubated overnight at 37°C.

Measurement of β-galactosidase activity. The measurement of β-galactosidase activity in each strain was performed as described (33).

RESULTS

We first describe the rationales of our screen for *Ind*⁻ mutants and for targeting the mutations to particular parts of the protein.

Basis of the screen. We took advantage of a constitutively activated mutant form of the RecA protein, termed RecA730, which cleaves wild-type LexA in vivo in the absence of SOS induction, greatly reducing its function. By contrast, noncleavable LexA(*Ind*⁻) proteins are resistant to cleavage and show greater repressor function. This difference was detected by the use of operon fusions between a LexA-controlled promoter and the *lacZ* gene. The logic of this method is similar to that developed by Mount (35) and later used by Gimble and Sauer (13) to isolate *Ind*⁻ mutants of phage λ. In our method, the *lexA* gene was present on a plasmid, and it was driven by the *lac* (or *tac*) promoter and controlled by the Lac repressor. The regulatory circuitry used is shown in Fig. 3. The use of the *lac* (or *tac*) promoter instead of the natural *lexA* promoter avoided complications due to autoregulation of the natural *lexA* gene (29) and allowed overproduction of the mutant proteins for biochemical analysis (L. Lin and J. W. Little, manuscript in preparation).

In our system, *lexA*(*Ind*⁻) mutants were identified by transforming plasmids carrying the mutagenized *lexA* gene into an indicator strain, JL1420, with the following components (Fig. 3). First, it carried a *lexA71::Tn5*(Def) mutation, so that LexA function was provided solely by the plasmid. Second, it had a *sulA211* mutation (12), allowing the cell to divide under the SOS-induced state. Third, it carried a *recA730* allele (23), which made a constitutively activated RecA protein. This avoided the use of a DNA-damaging treatment to activate RecA, since the mutations we sought would make the cells sensitive to DNA damage. Fourth, it carried a fusion of the LexA-regulated *sulA* promoter to the *lacZ* gene in a λ prophage, allowing us to assess LexA

FIG. 3. Regulatory circuitry of the indicator strain (JL1420) used to screen for *lexA*(*Ind*⁻) mutants. In strain JL1047, the *recA* allele is wild type. In strain JL1752, the *recA* allele is deleted.

function by a simple screen. Finally, it contained an F' plasmid with a mutant Lac repressor which was insensitive to inducer (termed *lacI^r*), so that the *lexA* gene on the plasmid was not induced by the lactose present in the MacConkey indicator plate, an event which would be harmful or lethal to the cells.

The behavior of control plasmids in this indicator strain showed that it could be used to distinguish *lexA*(Ind⁻) mutants from wild-type *lexA*. Cells harboring a plasmid with a *lacP::lexA3*(Ind⁻) gene formed white colonies on MacConkey plates, showing that the basal level of noncleavable LexA3 protein could efficiently repress the *sulA::lacZ* fusion. By contrast, cells with a plasmid carrying a *lacP::lexA⁺* gene formed red colonies with white borders. Although the wild-type protein was made at the same rate as was LexA3 protein in the previous strain (see below), it was promptly cleaved, leading to low repressor levels and a high level of β -galactosidase. This difference in phenotype therefore allowed us to isolate more Ind⁻ mutants by screening for white colonies in a background of wild-type red ones.

This screen allowed us to pick up only noncleavable LexA mutants that retained sufficient repressor function to repress the fusion, avoiding isolation of mutants that lost their cleavability simply because their overall structure was disrupted by the mutation. However, in quantitative assays, some mutants gave β -galactosidase levels greater than those given by a *lexA3* plasmid. To distinguish whether these values reflected poor repressor function or some residual cleavability, a *recA* mutant version of the indicator strain was later used to assess repressor function in the absence of cleavage (see below).

This screen only detects changes that affect the RecA-mediated cleavage reaction, since a functional RecA protein is absolutely required for in vivo cleavage (51). It is plausible, in principle (14), that mutants isolated by this screen may not bear defects in the in vitro autodigestion reaction.

Targeting of mutagenesis and mutant isolation. A tryptic fragment of LexA protein, comprising the carboxy-terminal two-thirds of the protein from Leu-68 to the end of the protein, Leu-202, has full activity for RecA-mediated cleavage and autodigestion in vitro (25). Therefore, this part of the protein must contain all of the functional sites required for both cleavage reactions. Mutagenesis was targeted mainly to several small restriction fragments in this part of the gene, allowing us to saturate the mutations in a small region. The location of a mutation was then confirmed by subcloning the previously mutagenized fragment into a wild-type vector, and the exact position of the mutation was identified by sequencing this fragment instead of the whole gene. Localized mutagenesis was achieved by use of several restriction sites in the *lexA* gene which are unique in the plasmid we used, including naturally occurring sites and four new silent sites (30) for the enzymes *Bam*HI, *Sal*I, *Bst*EII, and *Bgl*II which were introduced by site-directed mutagenesis (Fig. 2).

Localized mutagenesis of several intervals was carried out using hydroxylamine, formic acid, and hydrazine mutagenesis methods. Mutagenesis gave roughly 10 times more *lexA*(Def) mutants than *lexA*(Ind⁻) mutants (see Materials and Methods), as would be expected, since the latter should result from relatively specific alterations in the protein.

Sequence of *lexA*(Ind⁻) mutations and clustering of amino acid changes in three regions. We isolated 46 *lexA*(Ind⁻) mutants and determined their mutational changes by DNA sequence analysis. The sequence changes and the mutagenesis method used are listed in Table 2. By use of three mutagens, our mutant collection contains a broad spectrum

of mutations (Table 3). As reported elsewhere (9, 32, 37), hydroxylamine and hydrazine generated GC $\rightarrow$ AT transition mutations; by contrast, formic acid generated mostly G $\rightarrow$ T and A $\rightarrow$ T transversion mutations.

The positions of amino acid changes for these *lexA*(Ind⁻) mutations are shown on a linear map of the LexA polypeptide (Fig. 4). Several striking features are evident. First, among these 46 mutants, no Ind⁻ mutations were found on the N-terminal side of Gly-80, although many of the mutants were isolated from experiments in which the mutagenized DNA included the *Mlu*I-*Sal*I interval (13 independent mutations were sequenced) or the entire *lexA* gene (8 independent mutations were sequenced). This finding is consistent with the idea that only the carboxy-terminal two-thirds of LexA protein is involved in the cleavage reactions (25, 48).

Second, the mutations clearly cluster into three small regions of the polypeptide. Region 1 includes amino acid residues 80 through 85, region 2 includes amino acid residues 114 through 120, and region 3 includes Thr-154 and Lys-156. We believe that these clusters correspond to sites required for cleavage (see Discussion).

Finally, of the 12 amino acids affected by these mutations, 9 are conserved between LexA and the cleavable repressors of phages λ , P22, and 434 (45). Several λ cI(Ind⁻) mutations also affect conserved amino acids (Fig. 4; see also Discussion). Moreover, most of this conservation extends as well to the UmuD protein (39), a bacterial protein required for SOS mutagenesis, which has recently been shown to auto-digest and to undergo RecA-mediated cleavage, an event believed to activate this protein in vivo (S. E. Burckhardt, R. Woodgate, R. H. Scheuerman, and H. Echols, Proc. Natl. Acad. Sci. USA, in press; T. Nohmi, J. R. Battista, L. A. Dodson, and G. C. Walker, Proc. Natl. Acad. Sci. USA, in press; H. Shinagawa, H. Iwasaki, T. Kato, and A. Nakata, Proc. Natl. Acad. Sci. USA, in press). We conclude

FIG. 4. Location of Ind⁻ mutations in the LexA protein and homology with other cleavable proteins. The carboxy-terminal two-thirds of LexA protein is depicted. The SA119 (Ser-119 $\rightarrow$ Ala) and KA156 (Lys-156 $\rightarrow$ Ala) mutations were previously described (47). Residues conserved among LexA, UmuD, and repressors encoded by phages P22, 434, and λ are boxed for the repressors and underlined in the UmuD sequence. The λ Ind⁻ mutations were reported by Gimble and Sauer (13). Numbers next to the partial sequence for LexA and λ repressors indicate the position in the respective proteins. The nomenclature for designating mutational changes is as given in Table 2.

TABLE 2. Sequence changes and resulting amino acid substitutions of *lexA*(Ind⁻) mutants

Allele no. ^a	Mutation code ^b	Amino acid change ^c	Codon change	Mutagen ^d	No. of independent isolates
<i>lexA264</i> (SSal)	GV80	Gly-80 → Val	GGT → GTT	FA	1
<i>lexA261</i> (SSal)	GD80	Gly-80 → Asp	GGT → GAT	HA	1
<i>lexA266</i> (SSal)	VE82	Val-82 → Glu	GTG → GAG	FA	1
<i>lexA265</i> (SSal)	VM82	Val-82 → Met	GTG → ATG	HA, FA	2
<i>lexA262</i> (SSal)	AV83	Ala-83 → Val	GCT → GTT	HA	1
<i>lexA267</i> (SSal)	AT83	Ala-83 → Thr	GCT → ACT	HA, HZ	4
<i>lexA252</i> (SSal)	AΔ83	Ala-83 → Δ	GCT → —	Spont.	2
<i>lexA260</i> (SSal)	AT84	Ala-84 → Thr	GCC → ACC	HA	1
<i>lexA268</i> (SSal)	AD84	Ala-84 → Asp	GCC → GAC	FA	1
<i>lexA3</i> (SSal)	GD85	Gly-85 → Asp	GGT → GAT	HZ	1
<i>lexA253</i>	EK95	Glu-95 → Lys	GAA → AAA	Spont.	1
<i>lexA274</i> (SSal)	RP114	Arg-114 → Pro	CGC → CCC	FA	1
<i>lexA269</i> (SSal)	VF115a	Val-115 → Phe	GTC → TTC	FA	4
	VF115b	Val-115 → Phe	GTC → TCC	FA	1
		Val-133 → Val	GTG → GTT		
<i>lexA263</i> (SSal)	GE117	Gly117 → Glu	GGG → GAG	HA, FA	3
<i>lexA251</i> (SSal)	SL119	Ser-119 → Leu	TCG → TTG	Spont., HA	7
<i>lexA280</i> (SSal)	ML120	Met-120 → Leu	ATG → TTG	FA	1
<i>lexA259</i> (SSal)	TI154a	Thr-154 → Ile	ACC → ATC	HA, HZ	3
	TI154b	Thr-154 → Ile	ACC → ATT	HA	2
<i>lexA270</i> (SSal)	KR156	Lys-156 → Arg	AAG → AGG	FA	1
<i>lexA272</i> (SSal)	KM156	Lys-156 → Met	AAG → ATG	FA	5
<i>lexA271-281</i>	KN156/QH184			FA	1
<i>lexA271</i> (SSalBgl)	KN156	Lys-156 → Asn	AAG → AAT		
<i>lexA281</i> (SSalBst)	QH184	Gln-184 → His	CAG → CAT		
<i>lexA273-282</i>	KT156/EV170			FA	1
<i>lexA273</i> (SSalBgl)	KT156	Lys-156 → Thr	AAG → ACG		
<i>lexA282</i> (SSalBst)	EV170	Glu-170 → Val	GAA → GTA		

^a The silent sites in the mutant *lexA* allele are indicated by the letters following the allele number. S, Silent site, *Sal*, *SalI*; *Bst*, *BstEII*; *Bgl*, *BglII* site.

^b For the sake of clarity, all the mutations in this paper are represented by the following code: two single-letter amino acid abbreviations, of which the first letter indicates the wild-type residue and the second letter indicates the substitution in the mutant *LexA*, followed by the position of the substitution.

^c Numbers represent the position of substitution.

^d Mutagens: FA, formic acid; HA, hydroxylamine; HZ, hydrazine; Spont., spontaneous.

that these residues are conserved, at least, because of their involvement in the cleavage reaction.

Repressor activity and cleavage of mutants in vivo. Three strains, with similar regulatory circuitry for controlling LacZ expression (see Fig. 3), were used to assess RecA-mediated cleavage and repressor function in vivo. They differed only in their *recA* alleles, bearing either constitutively active (JL1420), wild-type (JL1047), or deleted RecA function (JL1752). As described elsewhere (28, 47), the level of β-galactosidase activity was a measure of the efficiency of LexA repressor function in the absence or presence of RecA-mediated cleavage. In strain JL1752, RecA-mediated cleavage was totally eliminated by the absence of the *recA* gene, so that the level of β-galactosidase activity was solely dependent on the repressor function, determined by the operator-DNA binding activity and the intracellular stability of the repressor, which might be affected by other degradative pathways, such as the Lon protease (24, 40). In strain JL1420, which carried a constitutively active RecA protein, the level of β-galactosidase activity was determined by both the cleavability and the intrinsic repressor function of the LexA repressor. In strain JL1047, which made wild-type RecA protein, the level of wild-type repressor was reduced somewhat by the basal rate of cleavage, presumably due to a low level of activated RecA present in the cell, resulting in intermediate levels of β-galactosidase (24, 28, 47).

The levels of β-galactosidase activity in these three strains harboring plasmids with either wild-type or mutant repressor genes are listed in Table 4. Control plasmids displayed the

expected pattern. In cells with no repressor function (carrying pBR322), the *sulA::lacZ* fusion was fully derepressed in all three strains. In the *recA* strain bearing plasmids with

TABLE 3. Mutation specificity of *lexA*(Ind⁻) mutants generated by different mutagen treatments

Mutagen ^a	Nucleotide change in the codon	Mutation ^b	No. of independent mutations
HA	Transitions		
	C→T	C→T	12
	G→A	C→T	7
FA (mp8-42)	Transversions		
	G→T	G→T	9
	A→T	A→T	7
	G→C	G→C	1
	A→C	A→C	1
FA (mp9-147)	Transitions		
	G→A	G→A	2
	A→G	A→G	1
	Transversions		
	T→A	A→T	1
HZ (mp8-42)	Transitions		
	C→T	C→T	1
	G→A	C→T	2

^a Mutagens: HA, hydroxylamine; FA, formic acid; HZ, hydrazine. mp8-42 and mp9-147 denote the use of single-stranded DNA of these phages as templates in the mutagenesis treatment.

^b Change in the base originally located in the mutagenized DNA.

wild-type repressor or the *lexA3*(Ind⁻) allele, the fusion was greatly repressed, and both *lexA* alleles conferred the same low level of *lacZ* expression, indicating that wild-type and LexA3 proteins have intrinsically the same repressor function (see also reference 47). By contrast, in cells with activated RecA, the plasmid with wild-type *lexA* gave much higher values, whereas the *lexA3*(Ind⁻) plasmid was essentially unresponsive to the *recA* allele (see also reference 47).

All of the mutant repressors, except the VE82, AΔ83, RP114, and SL119 proteins, provided nearly as much repressor function as did the wild-type repressor, as judged by the level of β-galactosidase in the *recA* mutant. Since the steady-state level of these four mutant repressors in strain JL1752 was the same as that of wild-type repressor in the same strain, as determined by Western blot (immunoblot) analysis (data not shown), their lack of repressor function is more likely due to defects in the intrinsic DNA-binding activity than to decreased stability of the mutant repressor in the cell.

In the *recA730* strain (JL1420), cells bearing wild-type repressor showed a greatly increased level of β-galactosidase activity as compared with that in the *recA* mutant, presumably as a result of the susceptibility of the repressor to constitutive cleavage by RecA730. By contrast, most of the mutants were essentially unaffected by the presence of RecA730 and showed levels of β-galactosidase 10- to 50-fold lower than that of wild type, as would be expected from the screening procedure. In the *recA*⁺ strain (JL1047), the wild-type repressor conferred an intermediate level, while most of the mutant repressors resulted in about the same

TABLE 4. β-Galactosidase activity in strains carrying various *lexA* alleles

Plasmid or mutation	Sp act ^a (U) in:		
	JL1420 (<i>recA730</i>)	JL1047 (<i>recA</i> ⁺)	JL1752 (<i>recA</i>)
pJWL147 (<i>lexA</i> ⁺)	3,000	700	70
pBR322	5,100	4,900	4,300
GV80	130	70	40
GD80	80	100	90
VE82	260	230	210
VM82	160	70	50
AV83	70	50	70
AT83	120	100	50
AΔ83	350	150	220
AT84	100	100	70
AD84	80	50	80
GD85 (<i>lexA3</i>)	70	150	90
RP114	800	520	520
VF115	130	150	140
GE117	120	150	110
SL119	1,300	1,200	1,200
ML120	140	150	60
TI154	190	120	70
KR156	70	50	40
KM156	60	100	60
KN156	100	90	70
KT156	90	100	70

^a β-Galactosidase activity of strains JL1420, JL1047, and JL1752 bearing wild-type LexA repressor (pJWL147), no repressor function (pBR322), or mutant LexA repressor from plasmid pJWL147 derivatives. The specific activity values (calculated as described in reference 33) are an average of duplicate samples in each of two separate experiments and contain a standard deviation of about 40%. These results were consistent with the colony color of the strains on MacConkey indicator plates, except that strain JL1420 carrying the TI154 mutant plasmid formed pink colonies while all other strains with β-galactosidase values in this range formed white colonies. This discrepancy may reflect physiological differences between cells grown on plates and in liquid culture.

FIG. 5. Survival curve of strain JL1420 bearing wild-type (□), TI154 (×), or KR156 (Δ) LexA repressor from *lacP::lexA* plasmid after mitomycin C treatment.

levels of β-galactosidase in the *recA*⁺ strain as in the *recA* and *recA730* strains.

In summary, a majority of the mutant proteins appeared to be normal as repressors and totally deficient in *in vivo* RecA-mediated cleavage, as judged both by the low level of β-galactosidase activity observed in the *recA* mutant and by the unchanged level in response to the state of RecA protein. A few mutations resulted in constitutively high values in all three strains, suggesting partial defects in repressor function. Finally, several mutations resulted in significant increases in β-galactosidase levels in strains with increased RecA function, suggesting that the mutant proteins retain some residual susceptibility to RecA-mediated cleavage (see Discussion).

Sensitivity of mutants to mitomycin C. A classical property of *lexA*(Ind⁻) mutants is their high sensitivity to DNA-damaging treatments (36), a reflection of their inability to derepress many genes in the SOS regulon. To test whether the mutant proteins were also able to prevent derepression of other LexA-controlled genes in addition to the fusion used in the screen, we measured the sensitivity to mitomycin C treatment of cells carrying the *lexA*(Ind⁻) mutations. Examples of survival curves for strains carrying plasmids with wild-type *lexA* and two mutations, TI154 and KR156, are shown in Fig. 5. Strain JL1420, bearing wild-type repressor, was resistant to mitomycin C treatment. Cells bearing the KR156 mutant repressor were very sensitive to the treatment, whereas cells bearing the TI154 repressor showed an intermediate sensitivity, consistent with the above evidence (Table 4) that the TI154 repressor retains some *in vivo* cleavability (see also the Discussion).

The sensitivity of all the mutants to mitomycin C treatment is summarized in Table 5. Most of the mutants showed <10% survival after treatment with mitomycin C for 60 min, implying that the mutant LexA repressors were resistant to inactivation by cleavage. However, in addition to TI154, cells bearing four mutant repressors, VE82, AΔ83, RP114, and SL119, showed greater than 10% survival after treatment. Taken together with data from the preceding section (Table 4), this finding suggests that these four mutant repressors cannot efficiently repress other SOS genes involved in DNA repair.

DISCUSSION

The results reported here show that at least 20 different amino acid substitutions in the LexA repressor result in a

TABLE 5. Mitomycin C sensitivity

Allele	No. of viable cells/60 μ l of dilution after mitomycin C treatment for:		% Survival
	0 min	60 min	
Wild type	2,000	1,600	80
GV80	1,360	8	0.6
GD80	2,300	2	0.1
VE82	720	380	52.8
VM82	640	40	6.4
AV83	1,400	2	0.1
AT83	1,200	80	6.7
A Δ 83	960	100	10.4
AT84	1,400	26	1.8
AD84	900	0	<0.2
GD85 (<i>lexA3</i>)	1,300	1	0.1
RP114	2,200	480	21.8
VF115	2,000	3	0.2
GE117	3,000	190	6.3
SL119	1,100	480	44.4
ML120	3,000	150	4.9
TI154	2,400	250	10.4
KR156	2,100	0	<0.2
KM156	1,600	0	<0.2
KN156	1,600	2	0.1
KT156	2,000	1	0.1

noninducible (*Ind*⁻) phenotype. That these mutant repressors bear specific defects in RecA-mediated cleavage was suggested by two lines of evidence. First, in strain JL1420, which has a mutant, activated form of RecA protein, each of the mutant LexA proteins repressed a *sulA::lacZ* fusion more efficiently than was the case with the wild-type repressor, indicating that the mutant repressor is more resistant to the cleavage mediated by RecA730. Second, all the *lexA*(*Ind*⁻) mutants were more sensitive to DNA damage, such as mitomycin C treatment, a typical *Ind*⁻ phenotype (36) which most likely arises from the inability of repressor to be inactivated through cleavage (29). More direct evidence for defects in cleavage has been obtained by the in vitro assay of RecA-mediated cleavage using purified mutant proteins or crude cellular extracts (Lin and Little, in preparation).

A general problem in characterizing mutant proteins is ascertaining whether the change alters the structure only locally or has widespread effects on the overall structure of the protein. In the absence of detailed structural information, a rapid and sensitive test is to measure a second function of the protein which is expected to be independent of the mutation (1). In our work, the screen used to isolate the mutants required that the mutant protein retain at least some repressor function; we then measured this function in the absence of cleavage in a *recA* mutant host. As with λ repressor (38), the operator-DNA binding activity of LexA repressor is primarily a function of the NH₂-terminal domain of the protein (19, 28). However, the portion of the protein containing the mutations, which comprises both the hinge region (connecting the NH₂- and COOH-terminal domains and including approximately amino acids 70 to 100) and the COOH-terminal domain, also plays a role in repressor function, perhaps through the dimerization of repressor or formation of the proper structure for contact between operator DNA and the NH₂-terminal domain of the repressor. A few of the *Ind*⁻ mutants did show partial defects in repressor function, confirming that these portions of LexA do play a role in this function.

Since most of the mutations appear to confer normal

repressor function (Table 4), we conclude that, for the corresponding proteins, the loss of cleavability in vivo is more likely due to a specific alteration in the protein required for the cleavage than to a general disruption of protein structure. Previous evidence (28, 47) suggests that this is also the case for other *lexA*(*Ind*⁻) mutations (*lexA3* = GD85, SA119, and KA156) affecting residues in all three regions of the protein in which our mutations lie (see Fig. 4). In vivo, all these mutants display normal repressor function by the same tests as those used here. In vitro, the mutant proteins purify like wild-type LexA, and they appear (with the possible exception of LexA3, which was not tested) to have wild-type melting temperatures, as judged by their susceptibility to thermolysin as a function of temperature. We have also found that all of the new mutant proteins tested purify like wild-type LexA (in preparation).

Over half of the mutant proteins appeared to have normal repressor function and to be completely defective in cleavage, and thus we may regard these as nonleaky (*Ind*⁻) mutants. However, the remainder of the mutations led to higher β -galactosidase levels in the *recA730* strain, and less sensitivity to mitomycin C, than shown by a nonleaky *Ind*⁻ mutant. We divided these mutants into two classes, based on their response to the *recA* allele of the host. Mutant repressors in the first class, including those encoded by VE82, A Δ 83, RP114, and SL119, were to some degree deficient in repressor function, as reflected in the high values of β -galactosidase activity in the *recA* mutant strain, in their unresponsiveness to the *recA* allele of the host, and in the higher fraction of survivors (>10%) after mitomycin C treatment. The second class, including VM82, ML120, TI154, and perhaps AT83 products, appear to retain some residual cleavability, since the repressor function of these mutant repressors decreased somewhat when activated RecA protein was present in the cell, and they conferred an intermediate sensitivity (1 to 10%) to mitomycin C treatment. Since both of these phenotypes were intermediate between those of wild type and nonleaky *Ind*⁻ mutants, we conclude that the rate of cleavage in these mutants is considerably less than in wild type. Finally, two other mutants (bearing GV80 and AT84) appeared to be somewhat leaky by one but not both of these criteria.

In addition to mutations that directly affect the cleavage reaction, the screen we used would in principle detect mutations that conferred stronger binding of LexA repressor to the *sulA* operator, either because a repressor dimer bound more tightly to the operator or because the mutant protein dimerized more readily. This is obvious for the case of tighter-binding repressors, but more difficult to address in the case of altered dimerization, because we do not know the in vivo dimerization constant. The only published value for the in vitro dimer dissociation constant, roughly 50 μ M (46), suggests that LexA dimerizes more weakly than any other repressor for which such a value is known (compare, for example, with the 20 nM value for λ cI repressor [14]). We discuss two plausible cases, for both of which we might expect increased dimerization to give greater repression.

In the first case, we assume that the in vivo dimerization constant is the same or similar to that seen in vitro. By comparison, the concentration of LexA in vivo when expressed from the natural promoter is about 1 μ M (34); we estimate from Western blots that the concentration of non-cleavable LexA when expressed in our system is roughly 5 to 10 μ M, whereas that of wild-type LexA is much less than 1 μ M. Under this assumption, the cellular concentration of dimers, and hence the extent of repression, would be highly

sensitive to even small changes in repressor levels—potentially an advantage for the SOS regulatory system, which needs to be highly responsive—and to small changes in the dimerization constant resulting from mutation. If this assumption is true, it is unlikely that such mutants are included in our collection. It is known (18; our unpublished data) that increasing the concentration of wild-type LexA leads to a much greater repression of *sulA* than that observed here. Therefore, increased repressor function would result in much lower values of β -galactosidase than seen in the wild-type case. However, none of the mutant repressors provided significantly better repressor function in the absence of RecA function than did the wild type (Table 4).

In the second, alternative case, we assume that LexA dimerizes more strongly in vivo, so that a substantial proportion of wild-type repressor is in the form of dimers. We also assume that, as with λ repressor (5, 8), dimers of LexA are resistant to RecA-mediated cleavage. Consequently, a mutation that leads to increased dimerization would result in a lower concentration of the monomer substrate, and the rate of cleavage would be reduced. If these assumptions are true, repressor function would not be very sensitive to increases in dimerization, and it is possible that our mutant collection includes mutations of this type. In vitro experiments should resolve the issue.

Our data suggest that the mutant repressors do not confer a specific alteration in binding to the *sulA* operator. The sensitivity to mitomycin C treatment of all of the mutants, as well as the parallel between partial sensitivity to mitomycin C and intermediate levels of *sulA* repression (whether conferred by low residual rates of cleavage or by partial defects in repressor function), indicate that these mutant repressors are affected in parallel with *sulA* in their ability to repress DNA-repair genes such as *recA*, *uvrA*, *uvrB*, and *uvrD*.

Our data also provide further insight into the organization of the LexA protein. The location of the *Ind*[−] mutations is consistent with the previous finding that a tryptic fragment of the LexA repressor, comprising from Leu-68 to the COOH terminus of the protein, has full activity in both the autolysis and RecA-mediated cleavage reactions, further confirming that all of the functional sites or structural determinants required for both cleavage reactions lie in the C-terminal two-thirds of repressor. This seems to be the case also for λ repressor, since a similar papain fragment has the same properties (25, 44, 48), and all the *Ind*[−] mutations in λ repressor occur within this region (11, 13, 22).

In this part of the protein, there is a weak homology (~13% identity) between LexA, the cleavable repressors of phages λ , P22, and 434 (45), and UmuD protein (39). In principle, these conserved residues could play roles in the common properties among these proteins, such as specific cleavage and repressor dimerization. In our *lexA*(*Ind*[−]) mutant collection, 12 amino acid residues have been changed; 7 of these are in conserved residues, and 2 others are in residues (84 and 115 in LexA) conserved in the phage repressors and for which UmuD protein has conservative changes (see Fig. 4). These data suggest that these conserved residues are important, at least, in the specific cleavage reaction. However, the remainder of the *lexA*(*Ind*[−]) mutations and two leaky *Ind*[−] mutations, EV170 and EK95, affect five nonconserved amino acids, suggesting that nonconserved residues may also play a role in the specific cleavage reaction.

It is likely that the catalytic mechanism and the specificity of the cleavage reaction, like those of the trypsin family (7), are determined by the conserved amino acids, whereas the

different rates in the cleavage reaction (25, 26, 41, 48) are the result of nonconserved amino acids that are utilized by each repressor for modulating the rate of cleavage. This phenomenon may occur through, for example, varied effects of different sets of amino acids in the stabilization of an intermediate step before cleavage, as proposed for the tyrosyl-tRNA synthetases found in the *Bacillus* family (50). Alternatively, the conformations of different sets of amino acids near the cleavage site may also determine the accessibility of the cleavage site to the catalytic center or the strength of interaction between the cleavage site and the active site. However, there is no direct evidence at this point to support any of the above mechanisms. Another possible role that may be played by the nonconserved amino acids is suggested by the *Ind*[−] mutations in λ repressor. Over half of these mutations affect nonconserved residues (not shown in Fig. 4) and are thought to be involved in the interaction with the RecA protein (14). This may also imply that the RecA interaction site is not conserved.

All the *lexA*(*Ind*[−]) mutations are clustered into three regions on the linear LexA protein sequence (Fig. 4). Region 1, including amino acids 80 to 85, contains the cleavage site. We have previously proposed (47) that regions 2 and 3, near Ser-119 and Lys-156, respectively, make up at least part of the active site, since mutations in Ser-119 and Lys-156 generated by site-directed mutagenesis produce proteins that are totally deficient in both types of cleavage reaction and are believed to be involved directly in the chemistry of cleavage. The present work adds further support to this view. Residues located near Ser-119 and Lys-156 also play important roles in cleavage; these residues may be involved in the actual chemistry or in the interaction of the cleavage site with the specificity portion of the active site. The present evidence cannot identify mutations specifically affecting various functional sites for the RecA-mediated cleavage; our biochemical analysis of mutant proteins (in preparation) addresses this question more directly.

Is our mutant collection saturated? We believe that it is likely to contain a majority of the mutations that can arise by single-base changes and will be detected in our screen, which requires not only a deficiency in RecA inactivation but also significant repressor function. Many mutants were isolated more than once after treatment with the same or different mutagens. One of these mutations, TI154, arose from two different changes in the same codon, ACC→ATC and ACC→ATT. Moreover, three classical *lexA*(*Ind*[−]) mutations, *lexA1*, *lexA2*, and *lexA3* (36), were all found in our mutant collection; *lexA1* contains the same change as GD80, *lexA2* is the same as TI154 (K. Peterson, N. Istock, and D. W. Mount, personal communication), and *lexA3* is the same as our GD85 (31). However, it remains likely that other amino acid substitutions requiring changes in two or three bases of the codon would also generate the *Ind*[−] phenotype.

The recently developed mutagenesis methods (37) we used here have the great advantage of generating transversions, a type of mutation that does not arise by most of the classical mutagenesis methods, thereby giving a broader spectrum of the possible mutations. For comparison, the λ cI(*Ind*[−]) mutant collection isolated by Gimble and Sauer (13) was generated by the use of mutagens giving mostly GC→AT transitions, such as hydroxylamine, nitrosoguanidine, and UV. By contrast, about half of our mutations are transversions. As a striking example, we isolated three different transversions and one AT→GC transition mutation in the critical residue Lys-156 of LexA repressor, whereas the corresponding lysine in position 192 of λ repressor, with its

AAA codon, could not be changed by mutagens giving GC→AT transitions. We would expect that changing this residue in λ repressor would also result in the Ind⁻ phenotype.

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