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Genetics

Rapid mutational analysis of regulatory loci in *Escherichia coli* K-12 using bacteriophage M13

(*lexA* operator/*recA* operator/*mutD5*/translational signal/gene fusion)

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ABSTRACT **A derivative of bacteriophage M13mp8, designated M13mp8/P, was prepared in which the promoter and NH_2-terminal codons of bacterial genes may be fused to a portion of β-galactosidase, resulting in an easily scorable phenotype. Because transcription from the inserted promoter remains responsive to the host regulatory system, it is simple to screen mutagenized phage for isolates with aberrant regulatory phenotypes and to determine the mutational changes by dideoxy sequence analysis. The feasibility of the method was demonstrated by isolation of a large number of mutations in the regulatory regions of two genes, *lexA* and *recA*. Base substitutions that altered the phenotype of recombinant phage were identified both in the single LexA repressor binding site of *recA* and in the two binding sites of *lexA*, as well as in other sites that likely affect translational efficiency. Our results suggest that this approach will be generally useful for mutational analysis of transcriptional and translational regulatory elements.**

Most systems of prokaryotic gene regulation operate by controlling the rate of transcription initiation. These mechanisms generally work by specific interactions between regulatory proteins and DNA binding sites near the promoter of the regulated gene. An important part of analyzing these specific interactions has been the characterization of *cis*-dominant binding-site mutations, which alter specific binding. These mutations are of interest for several reasons. First, they help to locate sequences that control gene expression. Second, they can be used to uncouple the expression of a gene from its regulatory system. Finally, they facilitate analysis of the chemical and structural nature of nucleic acid–protein interactions (1).

Most available binding-site mutations have been studied in systems in which it is easy to select or screen for the mutant phenotype. However, gene expression often does not lead to a scorable or selectable phenotype; in other instances, altering gene regulation might be deleterious to survival. In such cases (e.g., the SOS regulatory system), it would be desirable to find other more indirect means of isolating and characterizing regulatory mutations. We report here a rapid and versatile technique for this purpose.

Our method is an outgrowth of two recent technical developments. The first is a method for studying gene expression that makes use of gene and operon fusions. In this method (2, 3), regulatory regions are fused to the *lacZ* gene, which codes for the easily assayed β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23). A battery of methods has been developed for studying and manipulating the Lac system, so that it is one of the most versatile genetic systems. The second is the use of single-stranded phages such as M13 for rapid DNA sequence analysis by the dideoxy chain-termination method (4, 5).

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In our method, use is made of a phenomenon called α-complementation, in which the NH_2-terminal portion of β-galactosidase (α-donor peptide) can complement another mutation in *lacZ* impaired in this same part of the protein, to yield active β-galactosidase (6). An α-donor gene has been cloned into M13 by Messing *et al.* (7), yielding a family of phages that make plaque-like blue foci in a colorless overlay under suitable conditions. These phages are used to clone foreign DNA, using many different restriction enzymes, and phages with foreign inserts in the α-donor gene can be recognized because they make colorless plaques. We have altered one of these phages, M13mp8 (8), so that it can be easily used to clone regulatory regions, yielding α-donor gene fusions that make colored plaques. The intensity of blue color provides a simple and easily scored indicator of the level of expression from the inserted promoter. In this report, we apply this system to the mutational analysis of two genes, *recA* and *lexA*, which are both repressed by LexA repressor (9), but which have been largely refractory to mutational analysis because of their pleiotropic effects and the difficulty of assaying their expression.

MATERIALS AND METHODS

Strains and Plasmids. *Escherichia coli* JM103 Δ(*lac-pro*) *thi strA endA sbcB15 hsdR4 F' traD36 proAB lacI^q lacZ*ΔM15 was the host for preparation of M13 derivatives. Bacteriophages M13mp8 and JM103 were obtained from Bethesda Research Laboratories. JM103 was transformed with plasmid DNA by calcium chloride treatment (10), with subsequent selection for ampicillin resistance and proline prototrophy. The mutator strain, DM2525, was constructed from JM103 by transduction (11) with bacteriophage Plvir, which was previously grown on W3110 *zaf-13*::Tn*10 mutD5* (from R. Maurer). Strains containing the *mutD5* allele (12) were maintained on minimal medium, to minimize mutagenesis in stock cultures (13). DNA of plasmid pBR322 (14), and its derivative pJL4 (Fig. 1), was prepared as described (10). DNA of plasmid pJL42 (19) was provided by B. Markham.

Growth of Phage. Phage stocks, single-stranded DNA, and superhelical double-stranded DNA, replicative form I, were prepared from infected cell cultures as described (16). Mutagenized phage stocks were prepared by diluting an overnight culture of DM2525 in M9 glucose broth into double-strength YT broth (11), followed by incubation with aeration at 37°C. At a density of 1×10^8 cells per ml, phages were added to yield a multiplicity of infection of 0.1. Incubation was continued for 7 hr, when the cells were removed by centrifugation (typical titer, 5×10^{11} plaque-forming units/ml: of which 1% were Lac^- mutants). The α-complementation reactions of isolated plaques were indicated by hydrolysis of 5-bromo-4-chloro-3-indolyl-β-D-galactoside, which was included in the overlay agar as described (16). When plasmid-bearing strains were used for plating lawns, ampicillin (100 μg/ml) was included in the overnight culture, but it was excluded from the plate test. Mitomycin C was added to overlay agar at 0.5 μg/ml.

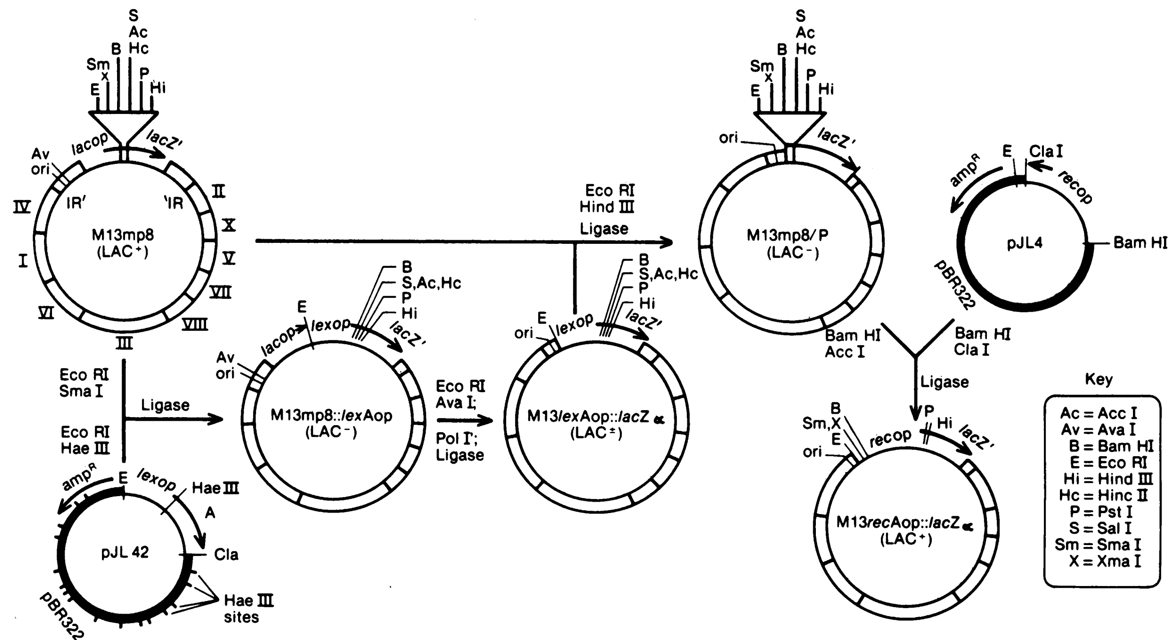

FIG. 1. Construction of M13mp8 derivatives carrying the *E. coli lexA* or *recA* promoters. Enzymes used are as indicated by letter codes (see key) and roman numerals identify M13 genes with IR being the M13 intergenic region. DNA fragment sizes are not to scale. Replicative form I DNA, used for *in vitro* recombination, was purified from infected cells by lysis and centrifugation in cesium chloride/ethidium bromide gradients as described (10), except that the lysozyme solution was 5 mg/ml. All enzymes were purchased from New England Biolabs and used as prescribed by the manufacturer, except as follows. *E. coli* DNA polymerase I (Klenow fragment, 2 units) was used to extend the recessed 3′ ends of restriction fragments by incubation of 5 μg of cut DNA in buffer (10 mM Tris·HCl, pH 7.6/2 mM $MgCl_2$/4 mM dithiothreitol/dATP, dGTP, dCTP, and dTTP at 80 μM each/10 μg of bovine serum albumin per ml) at 4°C for 10 min. All ligations were performed at a vector DNA concentration of 5 μg/ml with insert DNA (when applicable) at a 5- to 10-fold molar excess. Competent JM103 cells were transformed directly with the ligation reactions as described above, except that 0.1 ml of cells was added after the heat treatment and this mixture was immediately added to overlay agar and poured. In cases in which the phenotype of recombinant phage was not predictable, recombinant single-stranded DNA was screened for the expected restriction pattern (15). Phages that resulted from the insertion of the *lexAop* fragment were identifed by the ability of their DNA to hybridize (16) to a derivative of M13mp9 already known to contain this region of the *lexA* gene (unpublished observations). The plasmid pJL4 contains a 1-kilobase-pair chromosomal *Bam*HI/*Taq* I DNA fragment of the *recA* gene in place of the *Bam*HI/*Cla* I fragment of pBR322 (unpublished construction). The sequence of the *recAop* proximal end of this fragment is known (17, 18).

RESULTS

The objective of this work was to obtain a large number of regulatory mutants in the *lexA* and *recA* regulatory regions. The first mutants were obtained by cloning the *lexA* regulatory region with M13mp8. These experiments led to the preparation of an improved derivative of M13mp8 designated M13mp8/P, discussed below, which greatly facilitated a similar analysis of *recA*.

Fusion of the *lexA* Promoter to *lacZα* in M13mp8. We began by cloning a 116-base-pair fragment (*lexAop*), which includes the first 16 nucleotides of the *lexA* gene, its Shine–Dalgarno sequence (20), promoter, and repressor binding sites (21, 22) into M13mp8, in a construction that created an in-phase fusion of the *lexA* NH_2-terminal coding sequence to the eighth codon of the α-donor peptide gene of M13mp8 (see Figs. 1 and 4*A*). The existence of this fusion in five separate phages was confirmed by DNA sequence analysis. However, none of them displayed a detectable Lac reaction. The appearance of one phage (M13mp8::*lexAop*) on a Lac plate test is shown in Fig. 2 (streak 1). Since the addition of inducer (mitomycin C) to the plating tests did not increase the Lac reaction (data not shown), this lack of complementation was not due to repression of the *lexA* promoter by host LexA repressor. We considered two additional explanations for this result: (*i*) transcription from the *lacZ* promoter was inhibiting transcription from the *lexA* promoter, an effect called promoter occlusion (23); or (*ii*) the hybrid peptide was inactive or was rapidly degraded. Although the activity or stability of the hybrid peptide can indeed influence the strength of the color reaction, we showed that promoter occlusion may be in part responsible for the poor Lac activity of this phage. When a DNA fragment containing the *lacZ* promoter and translation initiation signals was removed from M13mp8::*lexAop* (Fig. 1), the resultant phage (M13*lexAop*::*lacZα*; streak 2 in Fig. 2*A*) displayed weak α-complementing activity.

Because the Lac phenotype of this phage was only marginally detectable, it was still not a suitable starting strain for the isolation of regulatory mutants. We therefore attempted to introduce mutations into the peptide that would improve

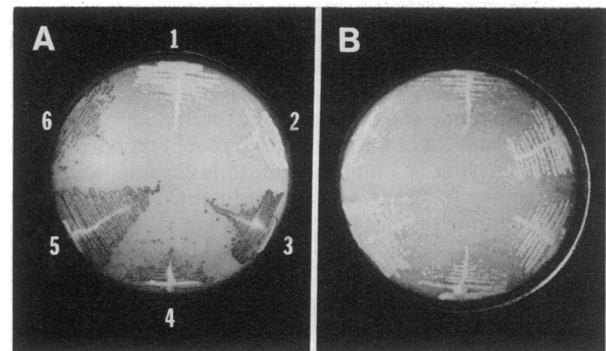

FIG. 2. α-Complementation activity of *lexAop*::*lacZα* fusion phage. Deeper tones represent a darker blue reaction on the plates, indicative of increased synthesis or activity of the α-donor peptide. Isolates are numbered the same in *A* and *B*: streak 1 is M13mp8::*lexAop*; streak 2 is M13*lexAop*::*lacZα*; streaks 3–6 are isolates a–d, respectively. (*A*) JM103/pBR322 and (*B*) JM103/pJL42 cells.

its α-donor activity. M13*lexAop*::*lacZα* was grown on the mutator host DM2525, and four isolates with increased α-complementing activity were obtained from 2000 progeny phages plaque-assayed on host JM103. The Lac reaction of the four mutants (a–d) is shown in Fig. 2*A* (streaks 3–6). One of these isolates (streak 5 in Fig. 2) had a G·C to A·T transition at position +53 in the first position of the eighth codon of the hybrid peptide (see Fig. 4*A*), predicting the substitution of isoleucine for valine. None of the other three had base substitutions within the region that was sequenced, from position +82 through the M13 gene II nicking site at position −122 (24). It seems likely that these three isolates have substitutions in the portion of the α-donor gene that was not sequenced.

To determine whether the α-complementing activity of M13*lexAop*::*lacZα* and its more reactive derivatives was dependent on transcription from the *lexA* promoter, we tested the Lac reaction of these phages on a host that harbored the multicopy *lexA* plasmid pJL42, which overproduces LexA repressor (unpublished observations). As shown in Fig. 2*B*, the α-donor activity of all these isolates was repressed.

Isolation of *lexA* Regulatory Mutants. To identify regulatory mutations in the *lexAop*::*lacZα* fusion, we utilized two observations made above. First, we reasoned that it should be easier to identify mutants if we started with a phage strain with a moderately intense Lac reaction so that increases could be detected. Second, we could avoid the repeated isolation of nonregulatory mutants if we screened mutagenized phage on a host that overproduces LexA repressor. Accordingly, the phage that gave the strongest complementation reaction, isolate d (see above), was grown on the mutator host, DM2525, and the progeny phage were tested for Lac activity on host JM103/pJL42. From 40,000 phages screened, 50 mutants that showed increased α-complementation were chosen. Nucleotide substitutions were observed in 24 of these isolates (see Fig. 4*A*). Eighteen of them had one mutation within the *lexAop* insert and one had two changes (positions −3 and +27). Among the 18 isolates, 15 had mutations that altered 5 different positions in the repressor binding sites with various effects on plaque phenotype. The other mutations lay within a region between +26 and +28, which has been identified as important for ribosome binding and translation initiation (25). Finally, 5 isolates had a single base substitution within the peptide coding region derived from phage M13mp8, and 3 of them had the same change at position +53 as isolate c described above. The other 2 had a change at position +52, which does not change the amino acid sequence.

Construction of the Promoter Cloning Vector M13mp8/P. To facilitate the construction of other gene fusions on M13, we replaced the *lexAop* fragment of M13*lexAop*::*lacZα* with the fragment in M13mp8 that contains six unique restriction sites, as shown in Fig. 1. The resultant vector M13mp8/P no longer carries a promoter, Shine–Dalgarno sequence, or initiation codon for the α-donor gene and is designed to serve as a cloning vehicle for *E. coli* promoters. This promoterless vehicle eliminates the problem of expression encountered when *lexAop* was cloned directly into M13mp8.

Insertion of *recAop* into M13mp8/P. We next tested whether M13mp8/P can be used to isolate regulatory mutations in the *recA* promoter, which is 10-fold stronger than the *lexA* promoter (unpublished observations). A DNA fragment of ≈1 kilobase pair containing the *recA* regulatory region (*recAop*) and the first three codons of *recA* was inserted into M13mp8/P. The resulting phage (M13*recAop*::*lacZα*) contained a fusion of the third codon of *recA* to codon 12 of the *lacZα* gene (see Fig. 4*B*), as confirmed by DNA sequence analysis. Although this phage demonstrated a weak complementation reaction (streak 1 in Fig. 3*A*), the phenotype was that expected of a *recAop*::*lacZα* fusion. The phage plaques

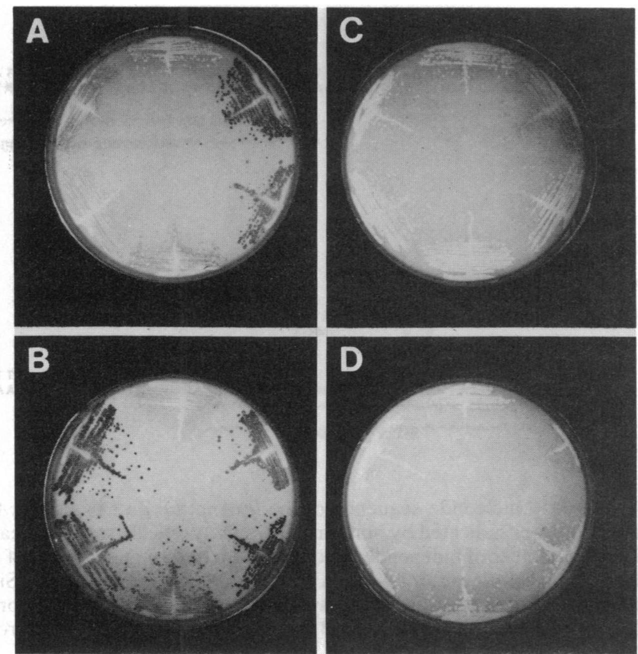

FIG. 3. α-Complementation activity of *recAop*::*lacZα* fusion phage. (*A*) JM103/pBR322 cells, (*B*) JM103/pBR322 cells with mitomycin C, (*C*) JM103/pJL42 cells, (*D*) JM103/pJL42 cells with mitomycin C. Isolates are numbered clockwise from the top, as in Fig. 2. Streak 1 is M13*recAop*::*lacZα*; streaks 2–6 are isolated a–e, respectively.

gave a stronger Lac reaction on medium with the inducer mitomycin C (Fig. 3*B*) and no detectable reaction in a host with high levels of LexA repressor (Fig. 3*C*).

Mutational analysis of the *lexAop*::*lacZα* fusion had demonstrated that both regulatory and α-peptide mutants can be obtained from a mutagenized phage stock. To test whether the same classes of mutants would be obtained from the *recAop*::*lacZα* fusion, the fusion phage was mutagenized as before and progeny were screened for mutants with increased complementation activity on JM103. Five representative independent isolates with the most distinct reaction were selected. Four of these phages (M13*recAop*::*lacZα*, isolates b–e; streaks 3–6 in Fig. 3, respectively) showed increased complementation activity with inducer in the medium (Fig. 3*B*) and were repressed in cells containing high levels of LexA repressor (Fig. 3 *C* and *D*). One of these four phages had a G·C to A·T transition at position +67 (Fig. 4*B*), while the others had no substitutions within the sequenced region from codon 10 of the *lacZα* gene to position −80. The remaining isolate (a; streak 2 in Fig. 3) had the insensitivity to the regulatory system expected of an operator constitutive mutant (Fig. 3 *B* and *C*) and an A·T to G·C transition at position −16 (Fig. 4*B*), within a LexA repressor binding site (21, 22).

Isolation of *recAo*^{c} Mutants. To isolate a large number of *recA* regulatory mutants, the mutagenized phage stocks described above were assayed on indicator host JM103/pJL42, which discriminated operator mutants from other types. In this case, it was not necessary to use a derivative phage with an improved α-donor peptide, because the *recAop*::*lacZα* fusion gave a strong enough initial reaction. We chose all plaques that appeared to show an increased Lac reaction in order not to miss any mutants with sequence changes that have a weaker regulatory effect; 40 variants were obtained from 10 independent phage stocks—a total of 7000 progeny plaques were screened. The phenotype of these isolates was tested on normal and repressor-overproducing hosts in the presence or absence of inducer. Nineteen of the original 40

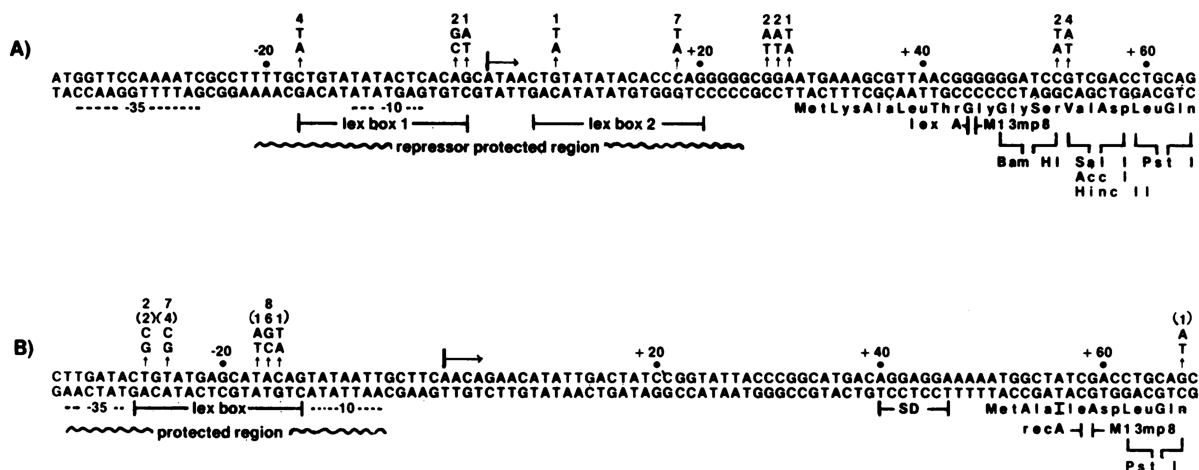

FIG. 4. Nucleotide sequence of the *lexAop::lacZα* (*A*) and *recAop::lacZα* (*B*) fusions. Sequences are numbered relative to their mRNA initiation sites indicated by horizontal arrows. Vertical arrows indicate position and identity of base-pair substitutions. Numbers in parentheses indicate number of independent isolates at each site; total number of isolates analyzed is shown above each site. Approximate regions protected from DNase I digestion by LexA protein (21, 22) are indicated. Single-stranded phage chromosomes were prepared and sequenced by the protocol of Sanger *et al.* (4). A synthetic 15-mer was used as a primer for polymerase elongation by hybridization to codons 11–16 in the M13mp8*lacZα* gene. [α-^{32}P]dATP (800 Ci/mmol) was purchased from New England Nuclear.

isolates demonstrated the phenotype predicted for a *recAo*^{c} mutant. All had a base substitution at one of five sites within the LexA repressor binding site in the *recA* operator (Fig. 4*B*).

DISCUSSION

We report the construction and use of M13mp8/P, which provides multiple unique restriction sites within the α-donor gene for cloning promoters and their associated regulatory sequences. Owing to the versatility of its cloning sites, several strategies can usually be devised for the formation of in-phase fusions to regulatory regions. Use of this phage also eliminates the expression problem posed by the *lacZ* promoter of phage M13mp8.

Our results test the general utility of this system in three respects. First, studies of the *lexA::lacZα* fusion demonstrate that mutations that derepress even weak promoters such as *lexA* (unpublished observations) can be detected. Second, because *recA* expression increases only 10-fold on derepression (26), the identification of operator constitutive mutants in this gene demonstrates that M13 fusions can be used to identify sites responsible for small regulatory effects. Finally, the stability and growth of the *recA* fusion phage indicate that the insertion of a strong promoter into the intergenic region of M13 has no deleterious effects on the phage.

Our nucleotide sequence analysis of mutant phage suggests that mutations that increase the α-complementing activity of a fusion phage are likely to act via three possible mechanisms: (*i*) by increasing the rate of transcription initiation of the α-donor gene; (*ii*) by increasing translation of the α-donor peptide by influencing ribosomal binding or mRNA stability; and (*iii*) by increasing the activity or stability of the α-donor peptide itself. Mutations that alter the nucleotide sequence of the LexA repressor binding sites (positions −17, −3, −2, +7, and +18 in *lexAop*; also positions −27, −25, −17, −16, and −15 in *recAop*) probably increase transcription by altering repressor binding. Although the possibility of subtle promoter effects is not excluded, substitutions at two of these positions are known to decrease repressor binding (unpublished observations; ref. 27). Substitutions in *lexAop* near the translation initiation codon (positions +26, +27, and +28) possibly act at the translational level (25). Mutations of this kind were not found among *recA* fusion mutants, presumably because we screened specifically for the *recAo*^{c} phenotype and because the *recA* ribosome binding site (17, 18) closely matches the Shine–Dalgarno consensus sequence. Mutations at +52 in the *lexAop::lacZα* fusion and +67 in the *recAop::lacZα* fusion do not change the sequence of the fusion peptide and likely increase translational efficiency or mRNA stability (28). Finally, one mutation that may increase the activity or stability of the peptide produced by the *lexAop::lacZα* fusion phage was observed at position +53. That this substitution is responsible for the improved α-donor phenotype of the phage was demonstrated by subcloning this region into M13mp8/P. However, we cannot be certain that this mutation does not also have a translational effect.

Phage mutants with different base substitutions in the LexA repressor binding sites in *lexA* and *recA* often showed different α-complementing activities, an effect that could reflect a difference in repressor binding affinity retained by the mutant site. However, it is important to rule out the existence of an additional mutation in the α-donor peptide gene, which might also influence the Lac activity of the phage. In this regard, we used phage M13mp8/P to reclone mutant operators or peptide coding sequences (positions −3, +18, and +53 in the *lexA::lacZα* fusion phage), and we have thereby proved that the identified base substitution is responsible for the mutant phenotype (*in vitro* backcross).

Although the mechanism whereby the mutations in the repressor binding sites of *lexA* and *recA* exert their effect is not yet known, some strong influences may be drawn. First, all mutations decrease dyad symmetry, suggesting a symmetric repressor–operator interaction. Second, mutations in the *lexA* operators change only the consensus sequence for repressor binding, whereas those in *recA* include additional changes that may be indicative of more repressor–operator contacts. Finally, since mutations were obtained in both binding sites in *lexA*, both sites are important for repression.

In conclusion, we have shown that gene fusions of phage M13mp8 are extremely flexible and powerful tools for the isolation of a large number of regulatory mutants of *E. coli* genes. A powerful aspect of this system is the ability to alter the α-complementing activity of the fusion peptide by *in vivo* mutagenesis to optimize conditions for the identification of regulatory mutations. Because these phages provide a ready source of single-stranded DNA, rapid nucleotide sequence analysis can be used to confirm the products of *in vitro* recombination or *in vitro* or *in vivo* mutagenesis.

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