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Detecting interactions between eukaryotic proteins in bacteria

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Few convenient genetic assays are available to study protein–protein interactions. This report describes a genetic scheme in E. coli to detect protein–protein interactions based on the concept of cooperative DNA binding of two interacting proteins. The yeast regulatory proteins GAL4 and GAL80, which are known to interact with each other, were used to test the scheme. A fusion protein, LexA-GAL80, was found to exert a cooperative effect on the DNA-binding activity of GAL4 as monitored by a bacterial repression assay.

Protein–protein interaction plays an important role in many biological processes. For example, gene activation in eukaryotes is thought to be achieved through interactions between activating sequences of transcriptional activators and a component of the general transcriptional machinery (Ptashne, 1988). The rate of transcription can also be modulated, either positively or negatively, by interactions among the regulatory proteins (for examples, see Mitchell et al., 1987; Chiu et al., 1988; Stern et al., 1989; Diamond et al., 1990). In yeast, the negative regulatory protein GAL80 inhibits the activity of GAL4, a transcriptional activator, by forming a complex with GAL4, masking its activating sequences (Ma and Ptashne, 1987b; Johnston et al., 1987; Lue et al., 1987).

Although many biochemical methods are available to study protein–protein interactions, there are few convenient genetic assays. Recently, two different genetic schemes have been described. In a yeast genetic scheme, the two essential domains of a eukaryotic activator, the DNA-binding domain and the transcriptional-activating domain, are carried on separate molecules that can interact with each other (Ma and Ptashne, 1988; Fields and Song, 1989). This yeast scheme should provide a general method to study protein–protein interactions, provided that the proteins to be studied are not themselves eukaryotic activators or components of the eukaryotic transcriptional machinery (see Discussion). In a second bacterial genetic scheme, λ phage repressor's dimerization domain, which is required for efficient DNA binding, was replaced by the dimerization motif leucine zipper from the yeast transcriptional activator GCN4 (Hu et al., 1990). This bacterial genetic approach, though successful for studying interactions between identical proteins, may not be best suited for studying interactions between different proteins. An additional method for studying protein–protein interactions (the phage surface protein display method) was recently developed; it combines genetic and biochemical approaches (Scott and Smith, 1990; Devlin et al., 1990; Cwirla et al., 1990; Bass et al., 1990; Clackson et al., 1991).

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This report describes a complementary genetic scheme in E. coli to detect protein–protein interactions based on the concept of cooperative DNA binding of two interacting proteins. The yeast regulatory proteins GAL4 and GAL80 (Johnston, 1987), known to interact with each other, were used to test the genetic scheme. The DNA-binding activity of GAL4 was monitored by a bacterial repression assay in which a GAL4 binding site overlapped the binding site for E. coli RNA polymerase: the binding of GAL4 repressed the bacterial promoter activity. A fusion protein, LexA-GAL80, which contains GAL80 fused to the DNA-binding protein LexA (Markham et al., 1981; Walker, 1984), was shown to enhance GAL4-mediated repression in E. coli. This bacterial genetic scheme could be used as a general method to study protein–protein interactions.

Materials and methods

Strains

The E. coli strains JM101(MA905A), JM101 (MA901), and JM101(MA942) contain in their chromosomes the *LacZ* gene under the control of the promoters 2XLex-UV5G4, UV5G4, and 2XLex-UV5P22, respectively. They were constructed according to Valenzuela and Ptashne (1989). Briefly, the plasmids pMA905A, pMA901, and pMA942 bearing the target promoters were first constructed according to standard procedures (Maniatis et al., 1982) and then recombined in vivo with the λRZ11 phage in the host strain NK5031(RZ11) (F^- *lacZ ΔMM5265 SupF* Nal^R $\lambda RZ11^+$) (Yu and Reznikoff, 1984). Phage lysates prepared after thermal induction of the host strain were used to transfect the E. coli strain JM101 (*SupE, Δlac proAB F' lacZ ΔM15 lacI*^{q}) (Yanisch-Perron et al., 1985) to generate λ lysogens bearing the proper target genes. In the absence of plasmids that express GAL4 or LexA derivatives, the β-galactosidase activities of 1477,2615, and 2335 were obtained from E. coli strains JM101(MA905A), JM101(MA901), and JM101(MA942), respectively. Promoters that contain identical "−35" and "−10" regions but different flanking or intervening sequences have been previously shown to have different activities (Paulmier et al., 1987; Lanzer and Bujard, 1988; Valenzuela and Ptashne, 1989).

Plasmids

Plasmids expressing GAL4 and LexA proteins were derived from pBR322 and pACYC184, respectively—two plasmids that are compatible with each other. The plasmid pMA910, which expressed the wild-type LexA protein from the *lac* UV5 promoter, was constructed by inserting the EcoR I-Hind III fragment of pJL184 (Lin and Little, 1988) into pACYC184. The plasmid pMA912, which expressed the LexA-GAL80 fusion protein from the *lac* UV5 promoter, was constructed in two steps. The fusion gene was first generated on the yeast vector LexA(202+PL) which contains a polylinker placed after the last codon (codon 202) of the *LexA* gene (Ruden et al., 1991). The *LexA-GAL80* fusion gene was then transferred to the E. coli vector pACYC184 by replacing the wild-type *LexA* gene in pMA910. The plasmid pSP80w, a gift from Peter Broad, which contains the coding region of GAL80 (residues 7–435) in the polylinker of pSP72, was used for the construction of the fusion gene. The amino acids that join the last codon of LexA and the seventh codon of GAL80 are Glu-Phe-Glu-Leu-Gly-Thr-Arg-Gly. The plasmids pSL20, pSL25, and pSL26, which were kindly provided by Stanley Liang, express from the *lac* UV5 promoter wild-type GAL4, GAL4(1–147), and GAL4(1–147) + I + II, respectively. The plasmids pMA930 and pMA931 were constructed by replacing the wild-type *GAL4* gene in pSL20 with the genes encoding GAL4(1–147) + II and GAL4 (1–147)–(851–881). The plasmids pTP8, pTP10, and pTP15, which expressed the P22 repressor at low, medium, and high levels (Poteete and Roberts, 1981), were kindly provided by Tony Poteete's laboratory.

Cell cultures and β-galactosidase assays

The E. coli cells were transformed with plasmids, and individual transformants were grown overnight to saturation in LB containing ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml) at 30°C. The overnight cultures were then diluted 200-fold in LB containing ampicillin and chloramphenicol. Thirty minutes after the dilution, IPTG was added to a final concentration of 1 mM, and standard β-galactosidase activity assays (Miller, 1972) were performed when OD_{600} of the cultures reached 0.8–1.2. The units of β-galactosidase were calculated using the following formula: Units = 1000 × OD_{420}/

$t \times v \times OD_{600}$, where OD_{420} is measured from the reaction mix (cell debris are removed by brief centrifugations), OD_{600} reflects the cell density just before the assay, t is reaction time in minutes, and v is volume of cell culture used in each assay in ml (normally 0.05 ml). See Miller (1972) for further details. Typically, three or four individual transformants were assayed for each sample, and the standard error was less than 20%.

Immunoblotting assays

E. coli cells were first transformed with two compatible plasmids expressing GAL4 and LexA derivatives. The cells were grown according to the procedure described above, collected, and lysed in loading buffer. The protein samples were then separated on 9% SDS-polyacrylamide gels and transferred to nitrocellulose filters. The amount of extracts loaded for each lane was equivalent to 0.08–0.2 ml bacterial culture, as normalized by cell density (Fig. 2A) or the intensity of the non-specific band (Fig. 2B). In the cases where duplicate lanes are shown, the extracts were prepared from two different bacterial cultures. The dilution of the antibodies—anti-LexA for the experiments in Figure 2A and anti-GAL4(1–147) for the experiments in Figure 2B—was 1/1000. The antibody reactions were visualized using ^{125}I-labeled protein A (New England Nuclear).

Color assay

The E. coli cells that were transformed with proper plasmids were streaked onto a nitrocellulose filter that had been placed on an LB plate containing ampicillin (50 μg/ml), chloramphenicol (34 μg/ml), phenethyl-thio-β-D-galactoside (tPEG; 0.5 mM–2 mM), and IPTG (0.25 mM). After overnight incubation at 30°C, the nitrocellulose filter was then transferred to an LB plate containing X-gal (50 μg/ml), as well as ampicillin, chloramphenicol, tPEG, and IPTG. For more information about tPEG, which is a competitive inhibitor of β-galactosidase, see Riggs et al. (1988) and the legend to Figure 3.

Results

Figure 1 schematically shows the design of the bacterial genetic system to study protein–protein interactions based on the concept of cooperative DNA binding. In this scheme, each of the proteins contains two domains: a DNA-binding domain and an "interacting" domain. Through contacts between the interacting domains, the

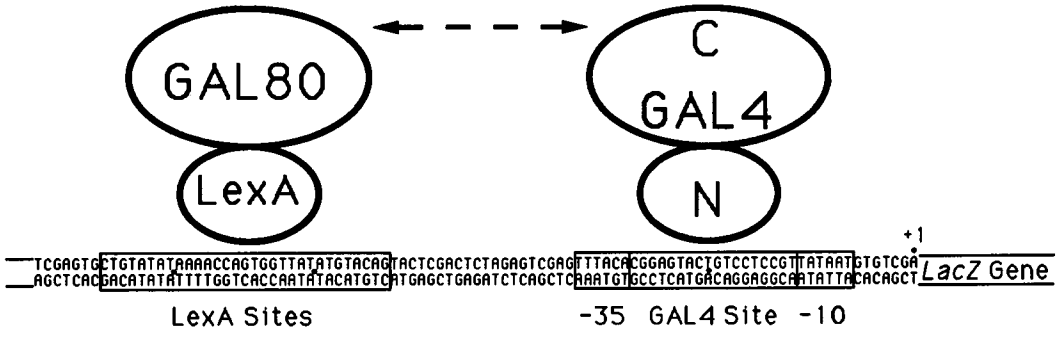


Figure 1. Experimental design. Each of the two proteins used in the cooperative DNA-binding test contains two domains. GAL4 contains an amino terminal (N) DNA-binding domain and a carboxy terminal domain (C) that interacts with GAL80 (the interaction is indicated by arrows). The LexA-GAL80 fusion protein contains the bacterial repressor protein LexA (residues 1–202; Markham et al., 1981) as the DNA-binding domain fused to GAL80 protein (residues 7–435 of the 435 amino acid protein; see Nogi and Fukasawa, 1984). The DNA-binding activity of GAL4 was monitored by a bacterial repression assay in which the GAL4 binding site was inserted between the "−35" and "−10" regions (Siebenlist et al., 1980) of the E. coli *lac* UV5 promoter (called UV5G4). To test the interaction between GAL4 and GAL80, two overlapping LexA binding sites found in the operator-promoter region of the E. coli colicin E1 gene (Ebina et al., 1983) were placed upstream of the UV5G4 promoter (called 2XLex-UV5G4 whose sequences are shown). In a control experiment, a different promoter (2XLex-UV5P22) was constructed that contained a P22 phage repressor binding site (O_R2) in place of the GAL4 binding site with the LexA binding sites located at the same position. These modified promoters, which controls the *lacZ* gene expression, were integrated into the E. coli chromosome as part of a λ prophage.

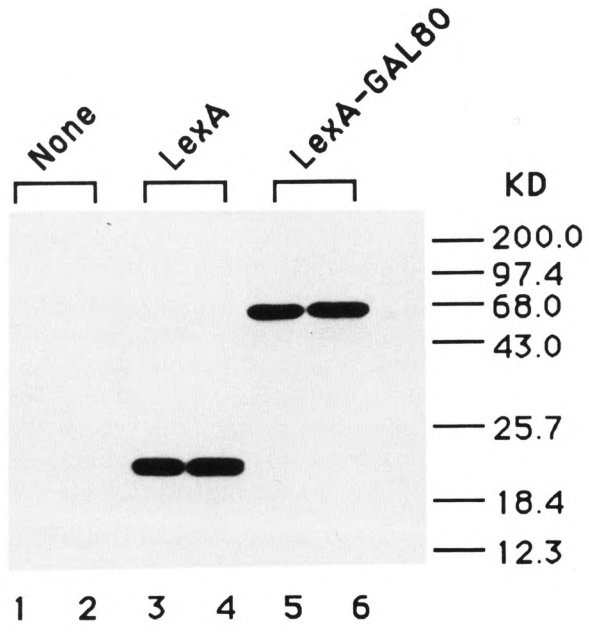
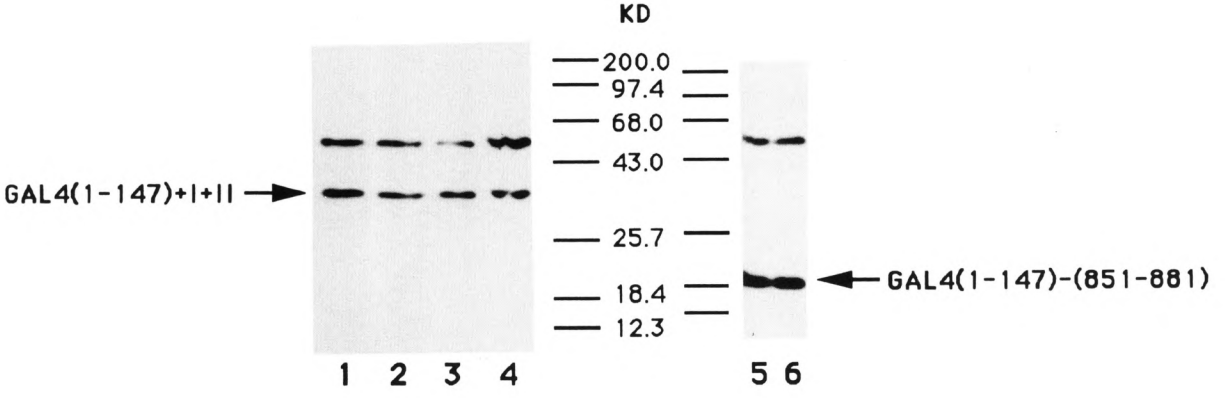


Figure 2. A. LexA derivatives. Shown are results of the immunoblotting analyses of proteins extracted from E. coli cells bearing plasmids that expressed either no LexA protein (lanes 1 and 2), wild-type LexA protein (lanes 3 and 4), or the LexA-GAL80 fusion protein (lanes 5 and 6). Note that the wild-type LexA protein and the LexA-GAL80 fusion protein were present in the cells at comparable levels. These levels of the proteins expressed from plasmids were much higher than that of the wild-type LexA protein expressed from the endogenous *LexA* gene (lanes 1 and 2); therefore the effect of the endogenous LexA protein on the assays shown in Table 1 and Table 2 should be negligible. The endogenous LexA protein (lanes 1 and 2), while invisible in this figure, could be seen on a longer exposure. **B.** GAL4 derivatives. Shown are results of the immunoblotting analyses of proteins extracted from E. coli cells bearing plasmids that expressed GAL4(1–147)+I+II (lanes 1–4) or GAL4(1–147)–(851–881) (lanes 5 and 6). These bacterial cells also contained plasmids expressing either wild-type LexA protein (lanes 1, 2, and 6) or the LexA-GAL80 fusion protein (lanes 3, 4, and 5). Note that the protein levels of the GAL4 derivatives were not affected by LexA-GAL80. A band of about 55kDa, which also appeared in the absence of any GAL4-expressing plasmid in the cells (data not shown), is probably a product of cross-reaction with the antibody against GAL4, and this band could be used as an internal control.



two proteins help each other bind to their respective DNA sites. The yeast transcriptional activator GAL4 contains both types of domains required for such an assay: a DNA binding domain at the amino terminus (N) and a domain at the carboxy terminus (C) that interacts with the negative regulatory protein GAL80 (Ma and Ptashne, 1987b, Johnston et al., 1987). Because GAL80 itself does not bind DNA (Lue et al., 1987), it was fused to the bacterial DNA-binding protein LexA (Markham et al., 1981; see Fig. 2A). When assayed in yeast, this LexA-GAL80 fusion protein, like wild-type GAL80 (Nogi and Fukasawa, 1984), inhibited the activity of GAL4 in the absence of galactose (not shown), indicating that the fusion protein retains GAL80's normal ability to interact with GAL4.

The DNA binding activity of GAL4 was monitored by a repression assay in E. coli. Although the yeast activator GAL4 also stimulates gene expression in other eukaryotes, including mammals, insects and plants, it exhibits no activating function in E. coli (S. Liang and M. Ptashne, personal communication), presumably due to differences in the transcriptional machinery of eukaryotes and prokaryotes (Ptashne, 1988, and references therein). However, the DNA-binding activity of GAL4 and its derivatives (Ma and

Table 1. The LexA-GAL80 fusion protein enhances GAL4-mediated repression in E. coli.

Assay	*Target promoter*	*GAL4*	*LexA*	*β-galactosidase units*	*Enhancement of repression*
1	2XLex-UV5G4	none	LexA	1,278	
2			LexA-GAL80	1,038	1.2
3		GAL4(1–147)+I+II	LexA	678	
4			LexA-GAL80	184	3.7
5		GAL4(1–147)+II	LexA	645	
6			LexA-GAL80	226	2.9
7		GAL4(1–147)–(851–881)	LexA	445	
8			LexA-GAL80	152	2.9
9		wt GAL4	LexA	803	
10			LexA-GAL80	356	2.3
11		GAL4(1–147)	LexA	124	
12			LexA-GAL80	123	1.0
13	UV5G4	none	LexA	2,373	
14			LexA-GAL80	2,216	1.1
15		GAL4(1–147)+I+II	LexA	1,236	
16			LexA-GAL80	1,350	0.9
17		wt GAL4	LexA	1,010	
18			LexA-GAL80	1,202	0.8

The enhancement of repression by LexA-GAL80 is calculated as a ratio of the β-galactosidase units in the presence of the wild-type LexA protein to the units in the presence of the LexA-GAL80 fusion protein. In the experiments shown above, two target promoters that direct the synthesis of β-galactosidase were used (see Fig. 1): 2XLex-UV5G4 (lines 1–12) and UV5G4 (lines 13–18). In addition to the wild-type GAL4 protein, the following GAL4 derivatives (Keegan et al., 1986; Ma and Ptashne, 1987a) were also used in the assays: GAL4 (1–147) is the DNA-binding domain of GAL4; GAL4(1–147)–(851–881) contains the carboxy terminal 30 amino acids of GAL4, a region sufficient to interact with GAL80 (Ma and Ptashne, 1987b; Johnston et al., 1987), fused to the DNA-binding domain; GAL4(1–147)+II contains the activating region II of GAL4 (residues 768–881) attached to GAL4(1–147); GAL4(1–147)+I+II contains both activating region I (residues 148–238) and region II fused the DNA-binding domain. In a different set of experiments, in which the LexA sites were moved 4 base pairs further upstream of the UV5G4 promoter, the enhancement of repression by LexA-GAL80 was detected, but the effects were less significant [2-fold for GAL4(1–147)+I+II]. LexA-GAL80 was also tested when a single LexA site was placed upstream of the UV5G4 promoter; the center–center distance between the LexA site and the GAL4 site is either 48.5 base pairs of 52.5 base pairs. The LexA-GAL80 fusion protein enhanced GAL4-mediated repression (2.9-fold for wild-type GAL4) only when the distance between the GAL4 site and the LexA site is 52.5 base pairs—when they are on the same side of the DNA helix (assuming 10.5 base pairs per helical turn). In the absence of LexA-GAL80, the magnitude of repression by the GAL4 derivatives correlates roughly with the amount of the proteins expressed in E. coli, as in the case of P22 repressor (see Table 2). For example, the repression by GAL4(1–147) was most prominent (line 11), and this smallest derivative of GAL4 was present at the highest level in the cells (data not shown). In the experiments shown in lines 1 and 2 of this table and lines 1 and 2 in Table 2, in which no GAL4 protein was present in the cells, the LexA-GAL80 fusion protein decreased the β-galactosidase activity slightly. This is probably a non-specific effect, because a similar slight difference in β-galactosidase activity was also observed, even when no LexA binding sites were positioned upstream of the target promoter (lines 13 and 14).

Ptashne, 1987a) can be easily detected in the following bacterial repression system. A GAL4 binding site was inserted between the "−35" and "−10" regions (Siebenlist et al., 1980) of the E. coli *lac* UV5 promoter (called UV5G4, Fig. 1). This modified E. coli promoter, which controls the *LacZ* gene expression, was repressed by GAL4 and its derivatives expressed in the bacterial cells (Table 1, lines 13, 15, and 17). Because the GAL4 binding site overlaps the E. coli promoter in this assay, DNA binding of GAL4 and its derivatives prevents or interferes with the binding of the E. coli RNA polymerase, thereby repressing transcription (Paulmier et al., 1987; Lanzer and Bujard, 1988). GAL4 and its derivatives did not repress E. coli promoters lacking the GAL4 binding site (not shown).

To test the interaction between GAL4 and GAL80, two LexA binding sites (Ebina et al., 1983) were placed upstream of the UV5G4 promoter (called 2XLex-UV5G4; Fig. 1); the center–center distance between the proximal LexA site and the GAL4 binding site is 42.5 base pairs. The LexA-GAL80 fusion protein helped GAL4 protein repress this modified E. coli promoter (Table 1, lines 1–10). For example, comparing the β-galactosidase activities obtained from cells bearing plasmids that expressed either the intact LexA protein or the LexA-GAL80 fusion protein (678 and 184 units respectively), the

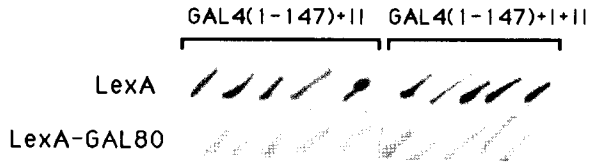


Figure 3. Color assays. The enhancement effect of the LexA-GAL80 fusion protein on the repression mediated by GAL4(1–147)+I+II and GAL4(1–147)+II can be observed on X-gal indicator plates containing 1mM tPEG (phenethyl-thio-β-D-galactoside), a competitive inhibitor of β-galactosidase (Riggs et al., 1988). While the cells bearing the plasmids that expressed LexA protein were blue, the cells bearing plasmids that expressed the LexA-GAL80 fusion protein were white or very light blue. The color difference between the bacterial cells was also observed on X-gal indicator plates containing 0.5 mM or 2 mM tPEG. In the absence of tPEG, all the cells were dark blue with no significant difference in shade on the X-gal indicator plates (not shown).

GAL4 derivative GAL4(1–147)+I+II repressed the E. coli promoter almost four times more effectively in the presence of the LexA-GAL80 fusion protein than in the absence of the fusion protein (lines 3 and 4). These differences in β-galactosidase activities can be easily detected on X-gal-containing indicator plates (Fig. 3). The enhancement of repression by LexA-GAL80 was also observed when the two LexA binding sites were moved 4 base pairs further upstream of the UV5G4 promoter, or when a single LexA binding site was placed upstream (see the legend to Table 1).

The following experiments suggest that the enhancement of GAL4-mediated repression by LexA-GAL80 is a specific effect resulting from the interaction between GAL80 and the carboxy terminal domain of GAL4. First, the enhancement of repression required the presence of LexA-binding sites positioned upstream: in the absence of the LexA binding sites no enhancement of repression by LexA-GAL80 was observed (Table 1, lines 13–18). Second, LexA-GAL80 did not enhance the repression mediated by GAL4(1–147), the DNA-binding domain of GAL4 lacking the carboxy terminal portion that is known to be required for the interaction with GAL80 (Table 1, lines 11 and 12). Third, as demonstrated by immunoblotting analyses (Fig. 2B), the LexA-GAL80 fusion protein did not increase the protein levels of the GAL4 derivatives in E. coli, suggesting that the enhancement of repression is not caused, for example, by stabilizing the GAL4 proteins in the bacterial cells. Finally, the LexA-GAL80 fusion protein did not enhance the repression mediated by P22 phage repressor (Table 2), which should not interact with GAL80. In these experiments, the LexA binding sites were placed upstream of a modified E. coli promoter (UV5P22; see Valenzuela and Ptashne, 1989) containing, instead of the GAL4 binding site, a P22 phage repressor binding site (O_R2) inserted between the "−35" and "−10" regions of the *lac* UV5 promoter (called 2XLex-UV5P22); the center–center distance between the proximal LexA site and O_R2 is 43 base pairs in the modified promoter.

Discussion

The experiments reported here demonstrate that the LexA-GAL80 fusion protein helps GAL4 repress the modified E. coli promoter 2XLex-UV5G4. This enhancement of repression is presumably achieved by increasing the DNA occupancy of GAL4 through contacts between GAL80 and the carboxy terminus of GAL4. It is also possible that a DNA loop formed between GAL4 and LexA-GAL80 may interfere with the binding of the E. coli RNA polymerase more effectively than GAL4 protein alone, thereby enhancing GAL4-mediated repression. The current experiments do not differentiate between these two possibilities. Although the enhancement of repression by the LexA-GAL80 fusion protein has never been greater than four-fold in the current tests, it is a reproducible effect, and the differences in β-galactosidase activities can be easily detected on X-gal-containing indicator plates (Fig. 3). The scheme described in this report is in principle an extension of the design of Valenzuela and Ptashne (1989), who showed that the binding of a P22 phage repressor to the modified E. coli promoter UV5P22 was increased by a second P22 phage repressor bound upstream (see also Martin et al., 1986; Hochschild and Ptashne, 1986; Flashner and Gralla, 1988; Haber and Adhya, 1988; Oehler et al., 1990). The current experiments suggest that the concept of cooperative DNA binding can be applied to studying interactions between eukaryotic proteins in E. coli. In contrast to the recently reported bacterial genetic assay (Hu et al., 1990) developed to study leucine zipper's dimerization properties, which are interactions between two identical proteins, the scheme described here was designed to study interactions between two different proteins.

Table 2. The LexA-GAL80 fusion protein has no effect on P22 phage repressor-mediated repression in E. coli.

Assay	*Target promoter*	*P22 Repressor*	*LexA*	*β-galactosidase units*	*Enhancement of repression*
1	2XLex-UV5P22	none	LexA	2,075	
2			LexA-GAL80	1,722	1.2
3		medium level	LexA	441	
4			LexA-GAL80	394	1.1
5		high level	none	91	

The promoter 2XLex-UV5P22, which directs the *lacZ* gene expression, was used in the experiments described in this table (see Fig. 1). The LexA-GAL80 fusion protein did not enhance the repression mediated by P22 phage repressor. The repression by P22 phage repressor correlates with the amount of the repressor made in the cells: while the repressor repressed the E. coli promoter less than twofold at low levels (not shown), it repressed the promoter more than 20-fold at high levels (line 5).

The current genetic scheme could be used as a general method to study protein-protein interactions. The two interacting domains tested here—GAL80 and the carboxy terminus of GAL4—can be replaced by two other interacting proteins (or domains). It should be pointed out that this bacterial genetic system may not be used successfully for proteins containing modifications that do not occur in bacteria but are necessary for protein-protein interactions. Because the current genetic system relies on repression, the concentration of proteins to be studied (the GAL4 fusion proteins in particular) should also be restricted to a relatively narrow range. In any case, this bacterial scheme complements a previously proposed yeast genetic method (Ma and Ptashne, 1988; Fields and Song, 1989), providing an alternative assay for proteins that are either harmful to or unstable in yeast cells, as well as for proteins that lack nuclear localization signals. A potential complication of the previously described yeast genetic method, which relies on gene activation, is that certain proteins or peptides to be studied may exhibit fortuitous activating functions in yeast: it has been demonstrated that eukaryotic activating sequences can be very readily generated (Ma and Ptashne, 1987c; Lech et al., 1988; Raycroft et al., 1990; Fields and Jang, 1990; Ruden et al., 1991). Components of the eukaryotic transcriptional machinery, when tested in the yeast genetic system, may also interfere with the assays. These complications can be avoided in the currently proposed bacterial genetic system, because eukaryotic activating sequences do not function in E. coli, and, furthermore, this bacterial system relies on repression rather than activation. Thus, interactions between (and among) eukaryotic regulatory proteins and components of eukaryotic transcriptional machinery, as well as other important protein-protein interactions, can be studied using this bacterial genetic system.

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