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Fused Protein Domains Inhibit DNA Binding by LexA

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Many studies of transcription activation employ fusions of activation domains to DNA binding domains derived from the bacterial repressor LexA and the yeast activator GAL4. Such studies often implicitly assume that DNA binding by the chimeric proteins is equivalent to that of the protein donating the DNA binding moiety. To directly investigate this issue, we compared operator binding by a series of LexA-derivative proteins to operator binding by native LexA, by using both in vivo and in vitro assays. We show that operator binding by many proteins such as LexA-Myc, LexA-Fos, and LexA-Bicoid is severely impaired, while binding of other LexA-derivative proteins, such as those that carry bacterially encoded acidic sequences ("acid blobs"), is not. Our results also show that DNA binding by LexA derivatives that contain the LexA carboxy-terminal dimerization domain (amino acids 88 to 202) is considerably stronger than binding by fusions that lack it and that heterologous dimerization motifs cannot substitute for the $LexA_{88-202}$ function. These results suggest the need to reevaluate some previous studies of activation that employed LexA derivatives and modifications to recent experimental approaches that use LexA and GAL4 derivatives to detect and study protein-protein interactions.

Chimeric proteins that contain the DNA binding domains of the bacterial LexA repressor (10) or the yeast GAL4 activator (36) have facilitated the study of transcription regulation. We and others have used the ability to separate DNA binding from other functions to identify and map activation domains (10, 30, 34, 43, 61, 65), ligand binding domains (20), and domains that interact with other proteins (16, 49, 54). LexA and GAL4 fusion proteins have also been used in screening procedures to identify activating motifs encoded by random bacterial open reading frames (50), to characterize the strength of activation domains of proteins in various cell types (34, 58), and to provide an internal standard in studies of site recognition by proteins that contain a second DNA binding domain (24, 33). Very recently, we and others have employed LexA and GAL4 derivatives as "baits" in interactor trap assays to identify proteins that complex with known proteins (12, 23).

LexA fusion proteins typically contain either the LexA amino-terminal DNA binding domain ($LexA_{1-87}$) (10, 45, 69) or the complete protein ($LexA_{1-202}$) which also includes a dimerization domain (4, 46, 69, 74). Like many prokaryotic repressors (26, 32, 56, 66, 67, 72), native LexA binds as a dimer to an operator that consists of two dyad symmetric half-sites (consensus sequence CTGTNNNNNNNNACAG) (5, 8, 77). LexA derivatives are assayed for transcription activation by using reporter genes that carry one or more LexA operators upstream of the transcription start site of a gene such as *lacZ* (for yeast assays) or CAT (for mammalian cell assays) (10, 20). For nonactivating LexA derivatives, DNA binding can be assayed by using a repression or blocking assay, in which binding of the LexA derivative to operator sequences located between an upstream activation site (UAS) and the transcription start site of a reporter gene diminishes its transcription (6, 9, 36).

One common use of LexA or GAL4 fusion proteins has been to compare the relative strength of activation domains between different proteins or between different deletion derivatives of the same protein. Implicit in such experiments is an assumption that heterologous fusion domains do not affect DNA binding by the LexA or GAL4 moiety; that is, the DNA binding moiety functions as an independent domain. However, in the course of conducting a detailed analysis of a series of LexA-Myc derivatives (22), we obtained results that suggested that this assumption is not correct.

Here we present a study of variables that affect operator binding by several different LexA derivative proteins. In these experiments we compared operator binding by native LexA to binding by a number of LexA derivatives, in vivo by using a transcription activation assay, and in vitro by using a gel mobility shift assay. We then examined the binding of a number of LexA derivatives to a set of mutant operators. The results show that many fused moieties dramatically reduce the ability of the LexA moiety to bind the LexA operator. They also show that the $LexA_{88-202}$ dimerization domain promotes high-affinity operator binding and that dimerization functions provided by heterologous proteins cannot substitute for this $LexA_{88-202}$-specific function. These results suggest the organization of native LexA is not strictly modular, in that the identity of one domain can affect the function of the other; in a simple model, a specific geometry of the $LexA_{88-202}$ dimerization domain encourages a spatially precise alignment of the $LexA_{1-87}$ domains on operators. We discuss the relevance of these findings for the use of LexA fusion proteins to study transcription regulation and for their use in recently developed methods to detect and study protein-protein interactions.

MATERIALS AND METHODS

Fusion proteins. Many of the constructions used in this study have been previously described; all fusions and reporters are represented schematically in Fig. 1. All constructions were made by using standard methods (1, 63). Fusion proteins were expressed from the strong constitutive ADH

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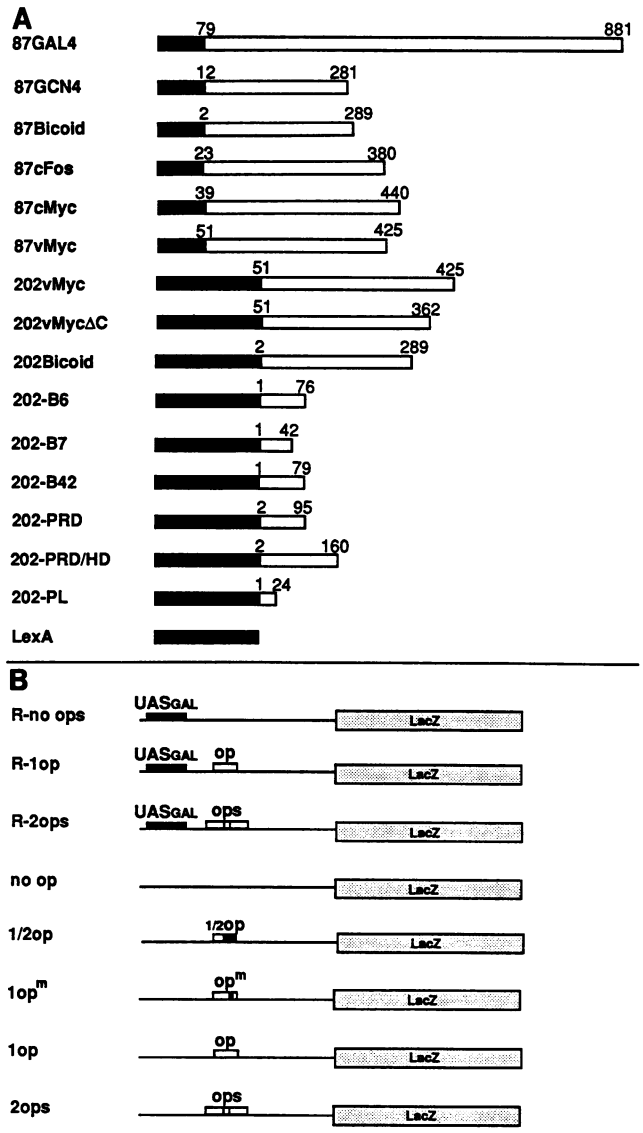

FIG. 1. (A) LexA derivatives. Black boxes in the figure indicate LexA sequences. Open boxes indicate fusion moieties. Numbers above open boxes indicate the residues from the native proteins included in each fusion protein. Most of the expression plasmids used in this study have been previously described; see Materials and Methods for details. (B) Reporter plasmids. The first three plasmids were used for repression assays; the next five were used for activation assays. Closed boxes indicate the $GAL4_{UAS}$. Boxes indicate the LexA operators, which are described in more detail in Materials and Methods. Open boxes, wild-type LexA operator; overlapping open boxes, overlapping or ColE1 operator, box with right half shaded, 1/2op; box with asterisk in right half, op^m.

promoter. The 2μm plasmid directing synthesis of $LexA_{1-87}$-GAL4 is RB1027 (10); the CEN ARS form is KL1027 (42). The 2μm plasmid expressing $LexA_{1-87}$-Bicoid is SH32-3 (24); the CEN ARS form and the 2μm plasmid expressing $LexA_{1-202}$-Bicoid (plasmid 14) are generous gifts of Steve Hanes (25). The 2μm plasmid expressing $LexA_{1-87}$-cFos is VR1001 (43); the CEN ARS form is KL1001 (42). The 2μm plasmid expressing $LexA_{1-87}$-cMyc is VR1004 (43); the CEN-ARS form is KL1004 (42). The 2μm plasmid expressing $LexA_{1-87}$-GCN4 contains residues 12 to 281 of GCN4 (28). The 2μm plasmid expressing $LexA_{1-87}$-vMyc is KA409 (43). The 2μm plasmid expressing LexA is pRB500 (8); cenLexA is the CEN ARS version (64). The 2μm plasmids expressing $LexA_{1-202}$-vMyc and $LexA_{1-202}$-vMycΔC contain full-length LexA fused to 1 to 415 and 1 to 362 of avian vMyc (57), respectively (22). The 2μm plasmids expressing $LexA_{1-202}$-B6, $LexA_{1-202}$-B7, $LexA_{1-202}$-B42, and their expression vector $LexA_{1-202}$-PL have been described (60). The 2μm plasmids expressing $LexA_{1-202}$-PRD and $LexA_{1-202}$-PRD/HD contain residues 2 to 95 encoding the PRD box and residues 2 to 160 encoding the PRD box and homeodomain of *bicoid* (3) fused to $LexA_{1-202}$ at the *Bam*HI site of $LexA_{1-202}$-PL (17).

Reporter plasmids. Repression reporters are as follows: the reporter plasmid *R-no op* is Δ20B (79). *R-1 op* is Δ20B + *1op* (9); it contains a single LexA operator cloned into an *Xho*I site between the GAL1 UAS and TATA of Δ20B. *R-2ops* is JK101 (33) and contains two overlapping LexA operators (as found upstream of the *colE1* gene [15]) in the Δ20B *Xho*I site. Activation reporters are as follows: the plasmid *no op* is LR1Δ1 (79). *1op* is 1840 (10, 43); it contains a single LexA operator inserted in the *Xho*I cloning site of LR1Δ1. $1op^m$ is a weak Oc mutation (75); it is identical to *1op*, except that in the LexA recognition sequence CTGTATGTACATACAGT, the G shown in boldface has been changed to a C (80). *1/2op* is also identical except that half the LexA operator has been destroyed by multiple base substitutions (CTGTATCTCGATATCC) (25). *2ops* contains the *colE1* LexA operators inserted into the *Xho*I site of LR1Δ1.

Expression and detection of fusion proteins. The *Saccharomyces cerevisiae* strain EGY40 (*ura3 trp1 his3 leu2*) was transformed by standard LiOAc methods (31). Expression of fusion proteins was monitored by Western immunoblotting (65) of 1 ml of mid-log-phase culture (optical density at 595 nm [OD_{595}], 0.6) by using anti-LexA antiserum (9) and alkaline phosphate detection (65). Absolute concentrations of LexA and derivatives were approximated from Western blots by comparison with serial dilutions of purified, quantitated LexA (8). This anti-LexA antiserum interacts preponderantly with the $LexA_{1-87}$ moiety (21), so the amount of fusion protein in extracts of $LexA_{1-87}$ derivatives could be accurately estimated by comparison to the amount of purified $LexA_{1-202}$ used as a standard. Intracellular and intranuclear concentrations of LexA derivatives were estimated by assuming a cell volume of 20 μm^3 (78) and an estimated nuclear volume of 2 μm^3 (9).

Activation and repression assays. The assay for activation was performed as previously described (10). β-Galactosidase values shown are the average of nine separate transformants, derived during assays performed on three separate occasions. Cells transformed with 2μm $LexA_{1-87}$-GAL4 and CEN ARS $LexA_{1-87}$-GAL4 were assayed both on glucose and on galactose. Results on both media were qualitatively identical, although actual β-galactosidase values were slightly lower on galactose media; values reported are those determined on glucose. As previously reported, $LexA_{1-87}$-GAL4 expressed from the ADH1 promoter activates on glucose because the C-terminal domain of GAL4 (41) is overexpressed sufficiently to titrate GAL80 (10, 49, 62).

The assay for repression was performed essentially as previously described (9). Values were determined for eight independent colonies, in assays performed on two separate occasions. Cells were grown on the appropriate selective media containing glucose to saturated overnight cultures; these cultures were spun down, washed with water, and

used to start cultures at an OD_{595} of 0.15 in galactose medium. Cultures were harvested at an OD_{595} of 0.5 to 0.6, and β-galactosidase values were determined. In both activation and repression assays, less than 25% variability was obtained between values for individual colonies expressing given fusion proteins.

Protein extracts. Cultures (500 ml) of yeast expressing appropriate fusions were grown to an OD_{595} of 0.6. Yeast cells were pelleted for 5 min at 3,000 × *g*, washed once in dH_2O, and resuspended in 5 ml of lysis buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 8.0], 5 mM EDTA [pH 8.0], 7 mM β-mercaptoethanol, 10% glycerol, 100 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 0.6 μM leupeptin, 1 μg of pepstatin A per ml). Glass beads (5 g) were added to each preparation; yeast cells were vortexed at high speed at 4°C for 15 min and then spun for 10 min at 4°C at 10,000 × *g* to remove crude debris. The supernatant was spun at 250,000 × *g* for 3 h at 0°C (50,000 rpm in an SW55); the resulting supernatant was concentrated ~20-fold in a Centricon-10 (Amicon, Inc.), aliquoted, and frozen at −70°C. Concentration and integrity of LexA and its derivatives were determined by probing Western blots containing 2 μl of each extract with anti-LexA antiserum as described above.

Gel mobility shift assay. The general outline of the protocol is that described previously (18, 19). Briefly, the 1op site, a double-stranded, blunt-ended oligonucleotide whose plus-strand sequence is 5'-AAAAGTACTA[CTGTATATACAT ACAG]TGATATCCCC-3', was used as a wild-type operator for binding in the assay; in this sequence, the LexA binding site is in brackets, and G · C base pairs whose G's are protected by bound LexA from methylation by dimethylsulfate are indicated in boldface (8). The $1op^m$ site, containing a single base change (shown in boldface) relative to the 1op site (CTGTATATACATACAC), was used in some experiments (see Results).

Binding reactions were done in 20 μl for 30 min at 30°C in a buffer composed of 4% glycerol, 1 mM EDTA (pH 8.0), 10 mM βME, 10 mM Tris-HCl (pH 7.6), 5 mM $CaCl_2$, and 100 mM NaCl to which extract was added to give a final fusion protein concentration either of 2 to 10 nM (~0.1 pmol per reaction) (Fig. 2) or of 0.2 pM to 2 nM (Fig. 3). In binding reactions, 20,000 to 50,000 cpm (~1 fmol) of γ-^{32}P-labelled oligonucleotide was used for binding, and 1 μg (~1,000-fold excess over labelled probe by weight) of double-stranded poly(dI-dC) was present to compete for binding by nonspecific DNA binding proteins. In addition, in competition assays, 100 ng (4 pmol) of the following unlabelled double-stranded oligonucleotides was also included in the binding reactions 1op; $1op^m$; Xop, which is identical to 1/2 except that the three DMS-protected G · C base pairs shown above in the LexA operator binding site have been altered to the bases shown in boldface (**ATATATATACATACAT**); or NS, a blunt-ended 30-mer that carried a consensus palindromic thyroid response element (GGGGATCAGGTCATGACCTG GATCCTCTAG) (52). Reactions were run on a 6% polyacrylamide gel (acryl-bis, 29:1 buffered in 0.25× TBE [1, 63]).

RESULTS

Many LexA derivatives bind operators poorly in vivo. We first wished to determine whether LexA derivatives bound tightly to their operators in vivo. In vitro, native LexA has a high affinity for naturally occurring operators (K_ds for binding to the operators used in our reporters range from of 2 × 10^{-10} M [*2ops*] [15] to 2 × 10^{-9} M [*1op*] [8, 9]). If LexA derivatives bound with similar efficiency, we would predict that even relatively low intracellular concentrations (~5 × 10^{-7} M) of these proteins should cause operators to be almost completely occupied; thus, increases above such levels of LexA derivative concentration would not cause increases in transcriptional activation. To measure binding in vivo, we expressed five different LexA derivatives from either low-copy-number (CEN ARS) or high-copy-number (2μm) plasmids in yeast cells (Fig. 1; Table 1) and measured activation of the *1op* and *2op* reporters.

Intracellular concentrations of the proteins were estimated from Western blot analysis of yeast cell extracts by using purified LexA as a standard (see Materials and Methods); calculated values are shown in Table 1. A number of the proteins (LexA-cFos, -Bicoid, -vMyc, and -cMyc) contain functional nuclear localization sequences (14, 27, 59, 73), and at least the LexA-cFos and LexA-Myc fusion proteins are nuclear localized in mammalian cells (22). Since the nuclear localization sequences of higher eukaryotes function in yeasts (53, 76), we expect that the intranuclear concentrations of these proteins should be approximately 5- to 10-fold higher than if they were uniformly distributed throughout the cell. We thus estimated that nuclear concentrations of the fusion proteins ranged from 0.02 × 10^{-6} to 2.8 × 10^{-6} M for proteins expressed from CEN ARS plasmids (low concentrations) to 0.7 × 10^{-6} to 9.0 × 10^{-6} M for proteins expressed from 2μm plasmids (high concentrations).

When expressed at low concentrations, all five LexA derivatives activated transcription poorly or not at all (Table 1). In contrast, at higher concentrations, all five proteins activated transcription, some more strongly than others. For example, $LexA_{1-87}$-cFos does not activate strongly at the lower concentration but does activate strongly at the higher concentration (Table 1). We do not know whether the activation observed at the higher concentrations reflects operator saturation, but we conclude from this result that at the lower concentrations operators are predominantly unoccupied. On the basis of the inferred nuclear concentrations, this suggests that LexA derivatives do not saturate operator binding at concentrations exceeding by at least 2 orders of magnitude those predicted to give half-maximal operator occupancy for native LexA. Native LexA binds operators more efficiently than LexA derivatives in vivo.

To test whether native LexA bound its operators with the predicted affinity in vivo, we used a transcription repression or blocking assay. This assay exploits the fact that LexA bound to either a single operator (*R-1op*) or two operators (*R-2op*) placed downstream of UAS_{GAL} blocks activation by endogenous GAL4 protein bound to the UAS (6, 9). We expressed LexA from CEN ARS or 2μm plasmids; these respectively directed LexA expression to presumed intranuclear concentrations of 8 × 10^{-8} M and 2 × 10^{-6} M, comparable to those of the LexA derivatives described above. In agreement with previous work, on a 2 operator target, native LexA repressed reporter genes by a factor of 20-fold, (Table 2), suggesting that even at the low concentration of LexA, less than 1 in 20 of the operators are unoccupied.

We also directly compared operator binding by a LexA derivative protein to that of native LexA by in vivo competition (Table 1). High levels of $LexA_{1-87}$-GAL4 (9 × 10^{-7} M) were expressed in cells that also expressed low levels of native LexA (8 × 10^{-8} M). In this experiment, because both proteins bind the same operators, the levels of activation of

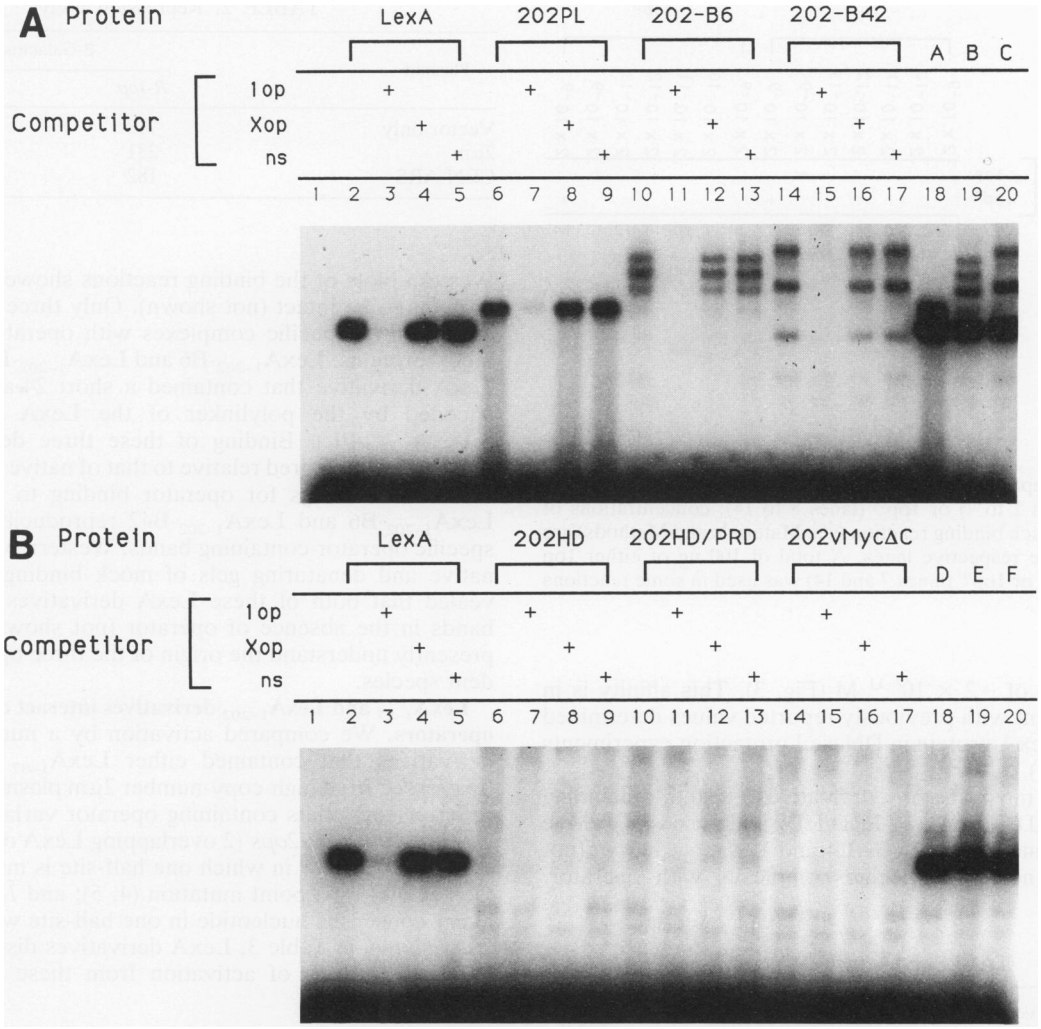

FIG. 2. Mobility shift assay of LexA and LexA derivatives. The 1op site was used as a probe in these experiments. Lane 1 in each panel contains unbound probe. All other lanes contain 2 to 10 nM the indicated LexA derivative: LexA (panels A and B, lanes 2 to 5 and 18 to 20), $LexA_{1-202}$-PL (panel A, lanes 6 to 9 and 18), $LexA_{1-202}$-B6 (panel A, lanes 10 to 13 and 19), $LexA_{1-202}$-B42 (panel A, lanes 14 to 17 and 20), $LexA_{1-202}$-HD (panel B, lanes 6 to 9 and 18), $LexA_{1-202}$-HD/PRD (panel B, lanes 10 to 13 and 19), and $LexA_{1-202}$-vMycΔC (panel B, lanes 14 to 17 and 20). The lanes designated A to C (A) and D to F (B) represent mixtures of the LexA extract with each of the other extracts used, to demonstrate that failure to obtain specific binding with some extracts was not due to the presence of inhibitors in the extracts; thus, A, LexA + $LexA_{1-202}$-PL; B, LexA + $LexA_{1-202}$-B6; C, LexA + $LexA_{1-202}$-B42; D, LexA + $LexA_{1-202}$-HD; E, LexA + $LexA_{1-202}$-HD/PRD; F, LexA + $LexA_{1-202}$-vMycΔC.

Lanes either contain no competitor (lanes 1, 2, 6, 10, 14, and 17 to 20) or 100 ng of specific 1op competitor (lanes 3, 7, 11, and 15), nonspecific Xop competitor (lanes 4, 8, 12, and 16), or nonspecific NS competitor (lanes 5, 9, 13, and 17). None of the specific bands observed competed with single-stranded specific 1op competitor (not shown).

the reporter gene should give a relative measure of the site occupancy by LexA and $LexA_{1-87}$-GAL4. We found that activation of the *2ops* reporter by $LexA_{1-87}$-GAL4 was completely blocked (<1 U) in the presence of native LexA, whereas in the absence of native LexA, the same reporter gene was activated by $LexA_{1-87}$-GAL4 to high levels (>1,000 U) (Table 1). Thus, even though $LexA_{1-87}$-GAL4 was expressed in substantial excess over LexA, LexA completely inhibited its ability to activate transcription. We interpret these results to mean that native LexA bound the operators with a far greater affinity than did $LexA_{1-87}$-GAL4.

Many LexA derivatives bind operators with low affinity in vitro. The experiments described above strongly suggested that LexA derivatives bound operator sites in vivo less well than did native LexA. This decrease in binding could indicate that LexA derivatives bound operators with lower affinity or that they were prevented from interacting with operators because they were sequestered by an interaction with some other cellular component (see Discussion). To distinguish between these ideas, we examined operator binding in vitro with a gel mobility shift assay (18, 19) by using whole cell extracts of yeast expressing different LexA derivatives. From Western blots, we estimated the concentration of LexA derivatives in these extracts to be at 2×10^{-9} to 10×10^{-9} M (not shown). A 36-bp double-stranded oligonucleotide that contained the 1op site was used to assay binding. Under these conditions native LexA bound 50% of the 1op site at a monomer concentration of $\sim 2 \times 10^{-9}$ M (Fig. 2) and bound detectable quantities of operator at a

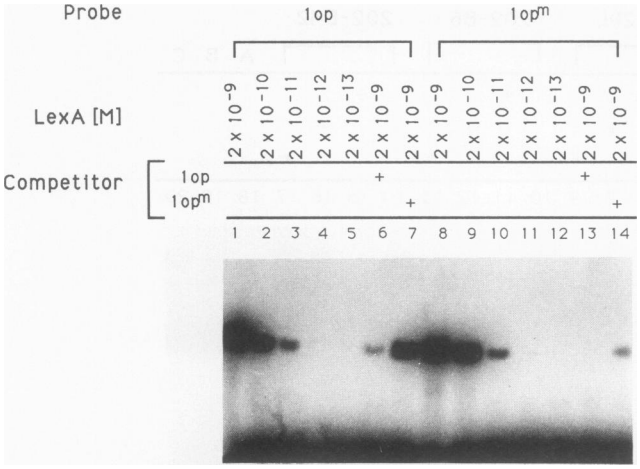

FIG. 3. Comparison of LexA binding to 1op and $1op^m$. Sites were 1op (lanes 1 to 7) or $1op^m$ (lanes 8 to 14); concentrations of LexA used in each binding reaction (see Materials and Methods) are given above the respective lanes. A total of 100 ng of either 1op (lanes 6 and 13) or $1op^m$ (lanes 7 and 14) was used in some reactions as a competitor.

concentration of $\sim 2 \times 10^{-11}$ M (Fig. 3). This affinity is in good agreement with previously reported values determined for purified LexA protein in DNase I protection experiments in vitro (4, 15).

In vitro, most LexA derivatives tested, including $LexA_{1-202}$-PRD, $LexA_{1-202}$-PRD/HD, and $LexA_{1-202}$-vMyc ΔC (Fig. 2), and $LexA_{1-87}$-GAL4 and $LexA_{1-202}$-vMyc (not shown), did not give specific complexes with operator.

TABLE 1. Activation assays[a]

LexA derivative and plasmid	β-Galactosidase (U)		Protein/cell (nM)
	1op	*2op*	
87GAL4			
2μm	1,152	1,192	900
CEN ARS	20	32	200
87Bicoid			
2μm	12	24	150^{NL}
CEN ARS	<1	<1	20^{NL}
87cFos			
2μm	780	1,000	900^{NL}
CEN ARS	<1	12	40^{NL}
87cMyc			
2μm	8	16	830^{NL}
CEN ARS	<1	<1	35^{NL}
202vMyc			
2μm	64	296	700^{NL}
CEN ARS	16	48	280^{NL}
LexA (native)			
2μm	<1	<1	2,000
CEN ARS	<1	<1	80
87GAL4 (2μm) + LexA (CEN ARS)	ND	<1	

[a] See Materials and Methods and Fig. 1. *NL*, contains nuclear localization signals; ND, not done.

TABLE 2. Repression assays

Plasmid	β-Galactosidase (U)	
	R-1op	*R-2op*
Vector only	718	324
2μm	231	33
CEN ARS	182	17

Western blots of the binding reactions showed these fusion proteins to be intact (not shown). Only three LexA derivatives formed specific complexes with operator: two "acid blob" proteins, $LexA_{1-202}$-B6 and $LexA_{1-202}$-B42 (60), and a LexA derivative that contained a short 24-amino-acid tail encoded by the polylinker of the LexA fusion vector ($pLexA_{1-202}$-PL). Binding of these three derivatives was only slightly impaired relative to that of native LexA, and we estimate their K_ds for operator binding to be $\sim 10^{-8}$ M. $LexA_{1-202}$-B6 and $LexA_{1-202}$-B42 reproducibly gave three specific operator-containing bands. Western blot analysis on native and denaturing gels of mock binding reactions revealed that both of these LexA derivatives ran as single bands in the absence of operator (not shown); we do not presently understand the origin of the three operator-dependent species.

$LexA_{1-87}$ and $LexA_{1-202}$ derivatives interact differently with operators. We compared activation by a number of LexA derivatives that contained either $LexA_{1-87}$ or $LexA_{1-202}$ (expressed from high-copy-number 2μm plasmids) of a set of reporter constructs containing operator variants. These reporters were *1op*; *2ops* (2 overlapping LexA operators [15]); $1op^m$, an operator in which one half-site is mildly disrupted (see below) by a point mutation (4, 5); and *1/2op*, in which every consensus nucleotide in one half-site was changed.

As shown in Table 3, LexA derivatives displayed several different patterns of activation from these reporters. All

TABLE 3. Activation assays on complex targets[a]

LexA derivative[b]	β-Galactosidase (U)			
	1/2op	$1op^m$	*1op*	*2op*
Class 1				
87Bicoid	ND	29	31	135
87cMyc	ND	<1	8	16
87vMyc	ND	<1	<1	52
Class 2				
87GAL4	3	60	1,152	1,192
87GCN4	<1	30	320	560
87cFos	<1	80	780	1,000
Class 3				
202vMyc	ND	72	64	296
202Bicoid	ND	121	93	135
202-B6	<1	168	196	1,748
202-B7	ND	148	260	860
202vMycΔC	<1	736	968	1,064
202-B42	<1	648	920	1,552
202-PL	ND	<1	<1	<1
LexA (native)	ND	<1	<1	<1

[a] The fusion proteins and reporters used are described in Materials and Methods and are shown in Fig. 1. ND, not done. No fusion protein activated the *no op* reporter, which lacks LexA operators (not shown).

[b] All derivatives were expressed from high-copy-number 2μm plasmids.

$LexA_{1-87}$ derivatives fell into two classes. Class 1 proteins ($LexA_{1-87}$-Bicoid, $LexA_{1-87}$-vMyc, and $LexA_{1-87}$-cMyc) activated all the reporters poorly and are discussed below. Class 2 proteins ($LexA_{1-87}$-GAL4, $LexA_{1-87}$-GCN4, and $LexA_{1-87}$-cFos) did not activate the *1/2op* reporter (with one exception) and activated the *1op*^{m} reporter only weakly. They activated the *1op* reporter strongly and the *2op* reporter only slightly more strongly. The exceptional class 2 protein, $LexA_{1-87}$-GAL4, activated the *1/2op* reporter extremely weakly but reproducibly (Table 3 and Discussion). All $LexA_{1-202}$ derivatives fell into class 3. None of these proteins activated the *1/2op* reporter. These proteins activated the *1op*^{m} and *1op* reporters moderately strongly and to comparable levels. Some class 3 proteins ($LexA_{1-202}$-B6, $LexA_{1-202}$-Bicoid, $LexA_{1-202}$-B7, and $LexA_{1-202}$-vMyc) activated the *2op* reporter substantially more strongly than the *1op*, while others ($LexA_{1-202}$-B42 and $LexA_{1-202}$vMΔC) did not; probably because expression of β-galactosidase directed by the latter proteins from the *1op* reporter was already saturated (1,200 to 1,500 U [21, 25]).

We then compared binding by native LexA to two of these sites in vitro (Fig. 3). Measured by 1/2 maximal operator binding, under our conditions the affinity of LexA for the $1op^m$ site differed only slightly from its affinity for the 1op site. In competition assays, the $1op^m$ site was 5- to 10-fold less effective as a competitor than the 1op site. However, by either assay, this mutation in the $1op^m$ site does not severely impair operator binding. Taken with our in vivo data, these results show that binding by LexA and LexA derivatives containing the $LexA_{88-202}$ domain is relatively insensitive to a weak operator mutation while binding by derivatives lacking this domain is significantly affected.

DISCUSSION

Operator binding by most LexA derivatives is impaired. Using transcription activation in yeast to monitor DNA binding in vivo and gel mobility shift assays to measure DNA binding in vitro, we found that operator binding by most $LexA_{1-87}$ and $LexA_{1-202}$ fusion proteins we examined is impaired relative to that of native LexA. This assertion is based on the following observations. First, fusion proteins that activated strongly when expressed at high levels did not activate (or did so very poorly) when expressed at lower concentrations, despite the fact that the estimated lower nuclear concentrations were as much as 2 to 3 orders of magnitude higher than the operator binding K_d for native LexA. Thus, at the lower concentrations, even though present in excess, the fusion proteins did not saturate their operator sites. Second, an in vivo competition experiment showed that $LexA_{1-87}$-GAL4, even when expressed at a 10-fold excess over native LexA, did not compete efficiently with native LexA for operator binding. Third, in vitro binding assays failed to detect binding to operator-containing oligonucleotides for many fusion proteins, including fusions to both $LexA_{1-87}$ and $LexA_{1-202}$, under conditions in which native LexA bound avidly.

Relative to binding of native LexA, even binding by most $LexA_{1-202}$ derivatives examined was substantially impaired (Table 3 and Fig. 2). It is worth noting, however, that two of the fused moieties we examined—vMyc and Bicoid—activated more strongly when they were fused to $LexA_{1-202}$ than to $LexA_{1-87}$ (Table 3); as we and others have argued, such differences in activation most likely result from improved operator occupancy conferred by the $LexA_{88-202}$ domain (33, 60) (see below).

The LexA carboxy-terminal dimerization domain specifically promotes operator binding. Many previous studies of LexA and LexA derivatives have suggested that high-affinity operator binding requires a dimerization function, which in native LexA is provided by the $LexA_{88-202}$ domain (8, 45–47, 60, 68, 69, 74). In one simple view, the enhancement of DNA binding contributed by the $LexA_{88-202}$ domain represents a dimerization-mediated increase in the local concentration of LexA molecules near an operator, such that binding of a first LexA protein to an operator half-site will automatically provide a "tethered" second LexA protein to fill the second half-site. In a second view, the $LexA_{88-202}$ domain contributes more actively to DNA binding by helping to position $LexA_{1-87}$ domains in a conformation required for stable binding on operator half-sites. The results of this study provide some support for the second view.

In the relevant experiments (Table 3), we compared operator binding by LexA derivatives in which a homodimerization motif was provided by native LexA sequences ($LexA_{1-202}$ fusions), was provided only by a heterologous moiety (for instance, $LexA_{1-87}$-GCN4 [29] and $LexA_{1-87}$-GAL4 [10]), or in which no strong dimerization motif was present (e.g., $LexA_{1-87}$-vMyc and $LexA_{1-87}$-cFos [55] [see below]). We assayed operator binding by examining activation by LexA derivatives of reporters that contained different operator variants. We reasoned that if the second view of operator binding described above was true, then in fusions that contain $LexA_{88-202}$ the energy gained from geometrically favorable monomer-monomer contacts between $LexA_{88-202}$ moieties might compensate for the energy lost from removal of a monomer-DNA contact; this might be revealed experimentally by an improved ability to interact with an operator with a weak mutation (*1op*^{m}). Our results supported this second interpretation. All fusion proteins that contained the $LexA_{88-202}$ domain (class 3) activated the *1op*^{m} and *1op* reporters similarly. In contrast, all fusion proteins that lacked the $LexA_{88-202}$ domain (class 2) were extremely sensitive to the *1op*^{m} point mutation (10- to 20-fold differences in activation), whether they contained other dimerization motifs or not. While this result does not exclude the possibility that heterologous dimerization motifs not examined in this study may in fact substitute for the $LexA_{88-202}$ domain, clearly not all do so.

These experiments also raise a related point. Heretofore, strong activation by $LexA_{1-87}$ fusion proteins has been taken to imply that the fused moiety contains dimerization sequences. However, the above experiments strongly suggest that all dimerization sequences are not equivalent in promoting operator recognition. Moreover, a survey of the published literature on LexA-activator chimeras indicates that such proteins may not need to contain any dimerization sequences, LexA specific or not, in order to activate. A large number of $LexA_{1-87}$ derivatives activate; at least some of the fused moieties present are either thought to contain weak dimerization sequences or are not known to contain them at all (22, 24, 27, 36, 56). Since it is unlikely that such proteins can quantitatively occupy operators, their abilities to activate may reflect the extreme sensitivity of the typical transcription activation assays, rather than their possession of dimerization sequences.

We note that although the LexA amino terminus has been shown to bind to isolated half-sites in vitro (37), our results do not generally support the idea that monomers of LexA derivatives bind to and activate from isolated operator half-sites in vivo. Most of the $LexA_{1-87}$ derivatives failed to activate the *1/2op* reporters, in which all consensus bases in

one half of the LexA operator were changed. One protein, however, $LexA_{1-87}$-GAL4, reproducibly gave very weak activation of this reporter. We suspect that this activation is caused by $LexA_{1-87}$-GAL4 dimers but that these dimers do not occupy the operator efficiently. It is also possible, however, that the activation we see reflects binding by an isolated monomer which activates inefficiently either because it does not bind a half-site well or because it does bind a half-site well, but multiple activation domains are necessary for substantial activation (i.e., synergy [12, 44]).

In sum, our results are most simply interpreted by postulating that the carboxy-terminal domain of a monomer of native LexA (and of $LexA_{1-202}$ derivatives) promotes a spatially precise association with another monomer on the DNA, in which the two amino-terminal DNA binding domains are optimally aligned in order to interact with their operator with high affinity. Lacking this precise protein-protein interaction, $LexA_{1-87}$ derivatives are much more sensitive to the removal of a single monomer-DNA contact in the *lop*^{m} site. Such a model for LexA binding is similar to recent proposals for binding by the glucocorticoid receptor (48). In addition, the idea that LexA monomers can associate on the DNA has recently received independent biochemical support (37).

Fusion domain-dependent interference with activation. We can imagine a number of possible mechanisms by which fusion domains might reduce activation by $LexA_{1-87}$ derivatives. Any or all of these may be reflected in the extremely low activation observed with class 1 proteins. First, the fused sequences might interfere with proper folding of the LexA moiety. We regard this possibility as unlikely, but in the absence of structural data we cannot exclude it.

Second, if the LexA derivative contains a second DNA-binding domain, the protein might be sequestered from LexA operators by binding to nonoperator DNA; such sequestration would be most severe in cells that had complex genomes. Sequestration on the DNA may well explain why the DNA binding region of the v-*rel* product inhibits activation by LexA-vRel derivatives in mammalian cells but not in yeast cells (33, 57), may account for lack of activation or inhibition of activation reported for the DNA binding domains of the Pit-1 (30) and Myc (22, 35) proteins, and may complicate the interpretation of studies of LexA-HMG derivatives, which also bind nonspecific DNA and which are reported not to activate at all (40).

Third, an oligomerization motif in the fusion moiety might cause the LexA derivative to form a complex with other cellular proteins that either keeps it from binding operator or allows it to bind operator but occludes the activation domain. Such complex formation might explain why, for LexA-Myc and LexA-Fos fusion proteins, removal of the oligomerization motifs (the helix-loop-helix/leucine zipper and leucine zipper, respectively) results in a 5- to 10-fold increase in their abilities to activate (Table 3) (22, 35, 42).

Fourth, the fusion moiety might sterically inhibit or otherwise increase the amount of energy required to position the LexA DNA binding domains properly on adjacent half-sites. Our in vitro data indicate that those fusion proteins least impaired for operator binding were those that contained relatively small fusion domains—the acid blobs B42, B6 (Fig. 3), and B7 (not shown) and the small moiety encoded by the polylinker of $pLexA_{1-202}$-PL. This last result raises the possibility that their relatively tighter binding contributes to the potency of acid blobs in transcription activation assays (50). In support of this, we have found that a LexA-VP16 fusion protein is a significantly stronger activator than a LexA-acid blob fusion (LexA-B112) when the proteins and a LexA-operator-CAT reporter are expressed at high levels in mammalian cells. However, when the two LexA fusion proteins are comparably expressed at low levels, LexA-B112 becomes a stronger activator than LexA-VP16 (21).

Consequences for future applications of fusion proteins. Our results suggest several considerations for use of LexA fusions. First, our data clearly show that operator occupancy in vivo differs greatly, both between different chimeric proteins and between identical chimeric proteins synthesized from different expression plasmids. In consequence, our results suggest that the degree of operator occupancy should be explicitly considered when different LexA derivatives are compared. In practice, the best way to ensure comparable occupancy will usually be to ensure that the different derivatives can fully occupy operators. Full occupancy is clearly favored by use of $LexA_{1-202}$ derivatives. Second, since saturation of β-galactosidase production occurs with very small numbers of DNA-bound strong activators (compare $LexA_{1-202}$-B42 with $LexA_{1-87}$-GAL4 and $LexA_{1-87}$-cFos [Table 3]), activators should be compared on a reporter that carries a small number of operators, which should ideally be positioned far enough (>200 bp) upstream of the *lacZ* gene to ensure that transcription is well below the maximum level.

Third, one of the more important applications of chimeric proteins in yeasts is as transcriptionally inert baits to detect interacting proteins from activation domain-tagged cDNA expression libraries (13, 17, 23, 70, 71, 80). Our data show that, at least when LexA derivatives are used for baits, two needs must be balanced. On one hand, many baits activate weakly, and it is important to saturate operator binding so that adventitious increases in bait expression in individual cells (21) cannot cause spurious activation of the reporter gene during the expression library screen. On the other hand, transcription of the reporter in a particular cell is dependent on the total amount of operator-bound bait that interacts with the activation domain-tagged protein encoded by a member of the expression library; for this to be maximized, the total concentration of bait should not exceed the total concentration of library-encoded protein. Our results suggest that these needs may be best met by the choice of $LexA_{1-202}$ rather than $LexA_{1-87}$ as a DNA binding domain and the use of short, nonactivating heterologous moieties in the bait. In addition, since native LexA lacks a nuclear localization sequence, gains in the degree of operator binding at low bait concentrations might come from the addition of a nuclear localization motif to baits.

Finally, although this study has confined itself to LexA derivatives, it is equally likely that some heterologous moieties can affect DNA recognition by other binding domains such as GAL4 (11). Given that the structures of GAL4 and LexA are quite different (2, 38, 39, 51), fused domains that are extremely deleterious to DNA binding in the context of LexA may be less so in the context of GAL4, and vice versa. Such DNA binding domain-specific effects may contribute to reported differences in activation strength between otherwise similar LexA and GAL4 derivatives (60).

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