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Transcriptional Repression by *Drosophila* and Mammalian Polycomb Group Proteins in Transfected Mammalian Cells

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The Polycomb group (Pc-G) genes are essential for maintaining the proper spatially restricted expression pattern of the homeotic loci during *Drosophila* development. The Pc-G proteins appear to function at target loci to maintain a state of transcriptional repression. The murine oncogene *bmi-1* has significant homology to the Pc-G gene *Posterior sex combs* (*Psc*) and a highly related gene, *Suppressor two of zeste* [*Su(z)2*]. We show here that the proteins encoded by *bmi-1* and the Pc-G genes *Polycomb* (*Pc*) and *Psc* as well as *Su(z)2* mediate repression in mammalian cells when targeted to a promoter by LexA in a cotransfection system. These fusion proteins repress activator function by as much as 30-fold, and the effect on different activation domains is distinct for each Pc-G protein. Repression is observed when the LexA fusion proteins are bound directly adjacent to activator binding sites and also when bound 1,700 bases from the promoter. These data demonstrate that the products of the Pc-G genes can significantly repress activator function on transiently introduced DNA. We suggest that this function contributes to the stable repression of targeted loci during development.

The establishment and maintenance of appropriate expression patterns during development requires a balance between negative- and positive-acting regulatory mechanisms. For example, many promoters are held in an inactive state despite the presence in the cell of activators that might otherwise turn on the promoter. The mechanisms by which transcriptional activity or inactivity is maintained must be stable and heritable from one cell division to the next. Extensive genetic analysis of *Drosophila melanogaster* has revealed that there is a set of factors necessary for maintenance of patterns of gene expression during development that is separate from the factors that function to establish that pattern (reviewed in references 4, 31, and 34).

The Polycomb group (Pc-G) genes are essential for maintaining a repressed state of numerous *Drosophila* genes. The mutant phenotype of the *Polycomb* (*Pc*) gene itself is homeotic transformation along the anterior-posterior axis of the *Drosophila* embryo, such that body segments take on an appearance appropriate to those which are more posterior (25, 31, 39). This phenotype is the result of ectopic misexpression of the homeotic control genes in the Bithorax complex (BX-C) and Antennapedia complex (ANT-C) (7, 24, 25, 50). Thus, despite the proper establishment of BX-C and ANT-C gene expression patterns, maintenance of these patterns is defective in *Pc* mutants (42, 44). All of the genes classified as Pc-G members share this mutant phenotype with *Pc*, leading to the hypothesis that Pc-G genes function coordinately to maintain spatially restricted patterns of expression by maintaining the repression of homeotic genes (9, 10, 21, 22, 42, 44). Double and triple mutants of Pc-G genes show synergistic interactions resulting in progressively more severe homeotic transformations, arguing that these gene products might function as a complex (1, 10, 22, 23, 29). There is physical evidence, as well, suggesting the existence of such a functional complex (13). Similar mechanisms might function in mammals, as indicated by the isolation of two mammalian genes with sequence similarity to the *Drosophila* Pc-G genes *Pc* (33) and *Posterior sex combs* (*Psc*) (5, 47). One of these genes, *bmi-1*, is similar to *Psc* over a 200-amino-acid (aa) region and was initially isolated as an oncogene, suggesting that it has an important regulatory role in mammals (17, 48).

It has been hypothesized that Pc-G proteins bind to specific sites and alter the local chromatin structure to repress gene expression. The proteins encoded by the Pc-G genes *Pc*, *polyhomeotic*, and *Psc* have been shown to localize to sites, many of which are identical, adjacent to known regulatory targets on polytene chromosomes (13, 26, 35). Large DNA segments from these target regions can confer Pc-G-dependent repression on a *lacZ* reporter gene (43, 52). These data suggest that the Pc-G members exert their regulatory effects by acting directly on target genes.

There are indirect lines of evidence that suggest that the targeting of Pc-G proteins leads to formation of a repressed chromatin state, perhaps resembling heterochromatin. The Pc protein shares an approximately 40-aa region of conservation (chromo domain) with the *Su(var)205* gene product, HP1, which is a heterochromatin constituent (32). The *Su(var)205* mutation suppresses position effect variegation, which is believed to occur because of heterochromatin formation (11). It has been proposed that HP1 functions to promote heterochromatin formation and that Pc and the Pc-G products function analogously (31, 36, 41). A separate line of genetic evidence also suggests a role for chromatin structure in Pc-G function: a mutation in *Pc* can be suppressed by *brahma* mutations (45). *brahma* has a high degree of sequence similarity to the yeast *SNF2/SWI2* gene, mutations in which are suppressed by mutations in the yeast core histone genes (18; reviewed in reference 51). These data have led to the proposal that *brahma* and other functionally related genes such as *trithorax* (23) function to relieve repression of genes by chromatin, while the Pc-G genes help establish a repressed chromatin state. While regulating chromatin structure is an attractive model for Pc-G function,

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there is no direct evidence that Pc-G proteins function via alterations in chromatin structure. Alternatives for Pc-G mechanism that are consistent with current genetic and structural data include the ability to block directly the function of nearby transcriptional activators.

To elucidate the mechanism by which Pc-G proteins repress expression, a functional analysis is required in addition to genetic and structural studies. As an initial step toward establishing a system for investigating the mechanism of Pc-G function, we determined whether Pc-G proteins and related proteins affected the functions of various transcriptional activators in a cotransfection system. We demonstrate that members of the Pc-G function as potent repressors in mammalian cells and show that the ability to repress an activator changes when the activation domain is changed. These data demonstrate that members of the Pc-G can regulate activator function and establish the basis for future studies on the mechanism by which Pc-G proteins function.

MATERIALS AND METHODS

Plasmid construction. Reporter plasmids containing LexA operators were generated as follows. LexA operator oligonucleotides were of the sequence 5′TGCTGTATATAAAAC CAGTGGTTATATGTACAGTACTG3′. This sequence corresponds to a ColE1 operator and contains binding sites for two LexA dimers (14). Complementary operator oligonucleotides containing appropriate sequences to generate specific restriction sites at each end were annealed and ligated. Only one of the oligonucleotides used in a given ligation reaction was phosphorylated at its 5′ end with T4 kinase, such that ligation would be directional and only dimers would form. Oligonucleotide dimers containing four operators were gel isolated, phosphorylated, and ligated into the various reporter constructs. "LexA operators" will refer to four operators in the following descriptions, unless otherwise indicated. (i) The reporter X523hspCAT was derived from 523CAT (38), which contains four copies of an array consisting of μE5, μE2, and μE3 from the immunoglobulin heavy-chain enhancer. LexA operators were inserted at a *Bgl*II site 10 bp 5′ of the nearest μE5 site to generate X523CAT. X523CAT was cut with *Bam*HI, blunt ended with Klenow enzyme, and then cut with *Nco*I. This released the promoter region 3′ of the 523 element and the 5′ end of the chloramphenicol acetyltransferase (CAT) gene. This was replaced with a *Sal*I (blunt)-*Nco*I fragment containing the human *hsp70* sequence from −40 (TATA region only) to approximately +160 and the identical 5′ portion of the CAT gene from pΔHS-40 (16). This generated X523hspCAT. The backbone of this construct is pSV2CAT, which has simian virus 40 (SV40) splice and polyadenylation signals 3′ of the CAT gene. (ii) XSHSE-CAT is based on SP72 (Promega), with the CAT gene between the *Eco*RI site and the distal end of the polylinker. A *Hin*dIII-*Nco*I fragment containing an extended heat shock element (15) at −34 of the human *hsp70* promoter and the CAT gene to the *Nco*I site was inserted into the corresponding sites of SP72CAT. LexA operators were inserted at the *Hin*dIII site to generate XSHSE-CAT. (iii) XG2CAT plasmids are based on plasmid G2-40hspCAT (46), which contains two *GAL4* sites (49) inserted with *Sal*I linkers at −40 of the human *hsp70* promoter. LexA operators were inserted at a *Hin*dIII site, which places them 20 bp 5′ of the *GAL4* sites; the construct containing six operators was the result of a cloning artifact. The resulting constructs were X4G2CAT and X6G2CAT. X2G2CAT contains two LexA operators and was generated by cleaving with *Bgl*II between the LexA operator oligonucleotide pair and *Sph*I, which cuts between *Hin*dIII and *Sal*I at the 5′ end of the *GAL4* sites. X300G2CAT is based on X6G2CAT and contains an approximately 300-bp fragment inserted at the *Sph*I site between the six LexA operators and the two *GAL4* sites. The insert corresponds to an *Nsp*I fragment from YEP24 (bp 5225 to 5519 [294 bp]; New England Biolabs, Inc.). X1700G2CAT was constructed in the same way as X300G2CAT except that the insert was an *Nsp*I fragment from the *Psc* cDNA (bp 3270 to 4941 [1,671 bp]) (5).

All expression constructs described below are based on CDM8 (40), the pertinent features of which are a cytomegalovirus immediate-early promoter, SV40 splice and polyadenylation signals, and an SV40 origin. CDMLex is the basic LexA expression plasmid and consists of a *Hin*dIII-*Eco*RI fragment encoding the full-length protein aa 1 to 202 from pL202PI (37), an *Eco*RI-*Sal*I fragment encoding the influenza virus hemagglutinin epitope tag (12), and the pSK(+) (Stratagene) polylinker from *Sal*I to *Not*I inserted between *Hin*dIII and *Not*I of CDM8. CDMLexBmi1 contains the *bmi-1* cDNA sequence from *Nae*I to *Pst*I, which encodes the full-length Bmi-1 protein with an additional 11 aa at the N terminus. The *bmi-1* sequence was inserted between *Sal*I (blunt ended) and *Pst*I. CDMLexSu(z)2 contains the *Su(z)2* cDNA sequence from *Nsi*I to *Nde*I inserted between *Pst*I and *Xba*I of CDMLex. The resulting fusion gene encodes all but the first amino acid of Su(z)2. CDMLexPc contains the *Pc* cDNA sequence from *Pac*I (blunt ended with T4 polymerase) to *Nsi*I, encoding the full-length Pc protein with one additional N-terminal amino acid. The *Pc* sequence was inserted between *Cla*I (blunt ended) and *Pst*I of CDMLex. CDMLexPsc contains the *Psc* cDNA sequence from the first *Nae*I (partial) to *Xba*I in the pKS(−) (Stratagene) polylinker inserted between *Sal*I (blunt ended) and *Xba*I. This *Psc* sequence encodes all but the first 11 aa of the Psc protein. CDMLexcdc2 was constructed by inserting a *Hin*dIII-*Sal*I fragment from a yeast expression plasmid (a generous gift of J. Gyuris and R. Brent) into CDM8 cut with *Hin*dIII and *Xho*I. CDMfBmi1 expresses the full-length *bmi-1* gene with the influenza virus tag at the N terminus. An *Nae*I-*Bgl*II fragment from the *bmi-1* cDNA was inserted into *Sal*I (blunt ended)-*Xba*I-cut CDMf, which is the same as CDMLex but without the LexA sequence. CDMf Su(z)2 and CDMfPc are identical in construction to their LexA fusion counterparts.

Mutant derivatives of LexA-Pc, LexA-Bmi1, and LexA-Su(z)2 were constructed as follows. LexA-Pc-ΔC86 was generated by cleaving CDMLexPc with *Nar*I in the *Pc* cDNA and *Xba*I in the 3′ polylinker, which removes sequences encoding the C-terminal 86 aa. Lex-Pc-ΔC118 removes sequences 3′ of *Bst*XI in *Pc*, which encode the C-terminal 118 aa. LexA-Pc-ΔC310 removes all but the N-terminal 80 aa and was generated by a cloning artifact. The chromo domain mutations, *XL5* and *LO35*, were generated by inserting PCR products generated from the corresponding mutant *Pc* cDNAs (kindly provided by R. Paro). CDMLexfBmi1-ΔN79 was generated by inserting a *Bst*1107I-*Xba*I fragment from CDMLexBmi1 into CDMLex (*Sma*I-*Xba*I). CDMLexfSu(z)2-ΔN removes aa 38 to 265 and was generated by deletion of the sequence between *Tth*III1 and the second *Bst*EII site (partial digest). CDMLexfSu (z)2-ΔC leaves only the N-terminal 298 aa and was generated by ligation of an *Nsi*I-*Aat*II (Klenow) fragment from the Su(z)2 cDNA into CDMLex (*Pst*I-*Xba*I-mung bean nuclease).

Activator expression vectors were the following: CDM8HEB (19); pBXG1, pBXG-AH, and pBXG-VP16, which encode the corresponding derivatives of GAL4(1–147) (kindly provided by M. Ptashne); pBXG-HSF1, which contains residues 201 to 529

of heat shock factor 1 (HSF1) (kindly provided by T. Schuetz); and pSG4+Sp1N, which expresses GAL4(1–147) plus Sp1 (83–621) (8), and p178, which expresses GAL4(1–147) plus c-Jun(5–253) (3) (kindly provided by G. Gill and R. Tjian).

Transient transfections and CAT assays. BALB/c 3T3 cells were used for all transient transfections for CAT assays. Cells were maintained in Dulbecco modified Eagle medium containing 10% calf serum on 100-mm-diameter tissue culture plates. Cells were split approximately 1:20 the day before transfection and fed 1 to 2 h prior to transfection. Transfection was by the calcium phosphate precipitation method (2). Total DNA per plate of cells was 21.5 μg; 0.5 μg of reporter plasmid, 4.0 μg of repressor expression vector, 4.0 μg of activator expression vector (except where indicated), 0.5 μg of human growth hormone (hGH) reference plasmid, and plasmid pSK(+) as carrier. All transfections and subsequent assays were performed in duplicate. Cells were washed and refed 16 h posttransfection and harvested approximately 48 h later. Harvesting of cells and phase extraction CAT assays were performed as described previously (2); briefly, cells were scraped from the dishes following a phosphate-buffered saline (PBS) wash, centrifuged, and resuspended in 200 μl of 250 mM Tris (pH 8.0). Cells were then subjected to three freeze-thaw rounds and heated to 65°C for 12 min. CAT assays were done in 100 μl at 37°C with 0.2 μCi of [^{3}H]chloramphenicol (32 Ci/mmol; NEN/DuPont) and 0.25 mg of *n*-butyryl coenzyme A (Sigma) per ml and stopped by extraction with 2:1 hexane-xylenes (2). The organic phase was removed to scintillation fluid for counting. Assays were done such that maximal sample activity was within the linear range of the assay: approximately 25% conversion, as determined by using purified CAT enzyme (5 Prime → 3 Prime, Inc.). CAT assay reactions for a given activator were allowed to proceed until the counts partitioning into the organic phase reached at least 5 and not more than 100 times the background counts (approximately 400 cpm) from a reaction containing lysate from untransfected cells. hGH radioimmunoassays were performed on media from transfected cells as instructed by the manufacturer (Nichols Institute). All CAT activity numbers are normalized to hGH (nanograms per milliliter) expression. The fold repression reported for the LexA repressors is expressed as the ratio of normalized CAT activity in the absence of repressor over normalized CAT activity in the presence of a given repressor. A potential artifactual explanation for the observed repression is that expression of the LexA repressors results in an increase in hGH expression, thereby lowering the normalized CAT activity number. This possibility was ruled out by the observation that in numerous repeat experiments, there is no increase in hGH expression in cells transfected with LexA repressor constructs relative to that of vector alone (data not shown). Some transfection experiments were normalized to β-galactosidase expressed from an SV40 promoter or a cytomegalovirus promoter. The results from these experiments were identical to those obtained with hGH used as a control for transfection efficiency (data not shown).

RNA analysis. Transfections of cells from which RNA was to be harvested were the same as those used for CAT assays, except that 4.0 μg of reporter and 8.0 μg of repressor expression plasmid were used and the transfected cells were harvested at 36 h posttransfection. Harvesting of RNA was performed as follows. Cells were lysed on their plates in 3.2 ml of 4.0 M guanidine isothiocyanate–25 mM sodium acetate–0.12 mM β-mercaptoethanol (GIT). Lysates were sheared four times with a 20-gauge needle and then layered on top of 2 ml of 5.7 M CsCl–25 mM sodium acetate in polyallomer tubes and spun at 32,000 rpm for 18 h at 20°C in a Beckman SW-55 rotor. RNA pellets were resuspended in 400 μl of 0.3 M sodium acetate, ethanol precipitated twice, and resuspended in water. Primer extension analysis was performed as described by McKnight et al. (27), with some modifications. Briefly, 50 μg of RNA was mixed in 20 μl with 2 ng of radiolabeled CAT oligonucleotide in 10 mM Tris (pH 7.5)–0.3 M NaCl–1 mM EDTA. The RNA-oligonucleotide mix was incubated at 75°C for 30 min, allowed to cool slowly to 50°C, and then incubated at 42°C for 16 h. Reverse transcription reactions were in a final volume of 80 μl containing 50 mM Tris (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM spermidine (Promega avian myeloblastosis virus reverse transcription buffer), 0.5 μg of actinomycin D per ml, 1.0 mM each deoxynucleoside triphosphate, 20 U of RNasin (Promega), and 16 U of avian myeloblastosis virus reverse transcriptase (Promega). Reaction mixtures were incubated at 42°C for 1 h, ethanol precipitated, resuspended in formamide loading buffer, and analyzed on a 5% denaturing polyacrylamide gel. S1 nuclease analysis (2, 15) was done with a 75-mer oligonucleotide complementary to the human *hsp70* 5′ untranslated region and spanning the site of transcription initiation. Hybridization was done in 80% formamide, and reaction products were analyzed on a 10% denaturing polyacrylamide gel.

IP. COS cells were used to overexpress GAL4 activators and LexA fusions from SV40 origin-containing vectors. COS cells were cultured in Dulbecco modified Eagle medium containing 10% calf serum; 5 μg of total DNA was transfected by the DEAE-dextran-chloroquine method (2). Transfection mixes were left on the cells for 4 h; then cells were shocked with 10% dimethyl sulfoxide–PBS for 2 min, washed with medium, and incubated for 16 h at 37°C. Cells were then suspended in 0.5 mM EDTA–PBS, centrifuged, trypsinized in 0.5 ml, and replated on the same dishes. At 72 h posttransfection, duplicate plates of cells were washed with PBS, pooled in 1.5-ml Eppendorf tubes in 500 μl of minimal essential medium lacking Met and Cys, and incubated at 37°C for 30 min. Then 70 μCi of ^{35}S-Trans Label (70% Met, 15% Cys; 1,037 Ci/mmol; ICN) was added to the cells, and labeling proceeded for 2 h at 37°C. Cells were washed three times in 1 ml of PBS and then resuspended in 500 μl of radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris [pH 7.4], 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulfate [SDS], 0.5 mM EDTA [pH 8.0], 75 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg each of aprotinin, leupeptin, and pepstatin A per ml) plus 10 U of RQ DNase (Promega). Cells were vortexed vigorously, incubated at 4°C for 15 min, and then spun at 32,000 rpm for 30 min at 4°C. The supernatants were collected and used immediately or frozen in liquid nitrogen. Equivalent amounts of trichloroacetic acid-precipitable counts were used for each sample to be immunoprecipitated. Labeled cell lysates brought to 400 μl with RIPA buffer were incubated with 10 μl of 50% protein A-Sepharose (Pharmacia)–PBS at 4°C for 30 min with gentle mixing. The protein A-Sepharose was pelleted, and the supernatant was collected and incubated at 0°C for 90 min with a polyclonal antiserum raised against GAL4 (1:200 dilution; kindly provided by M. Ptashne) or LexA (1:200 dilution; kindly provided by R. Brent); 25 μl of 50% protein-A Sepharose was then added, and the immunoprecipitation (IP) proceeded for 60 min at 4°C with gentle mixing. Samples were spun briefly, and the pellets were washed twice with 500 μl of RIPA buffer. IP complexes were dissociated in 30 μl of 2× SDS sample buffer at 100°C for 5 min and then separated on an SDS–10% polyacrylamide gel.

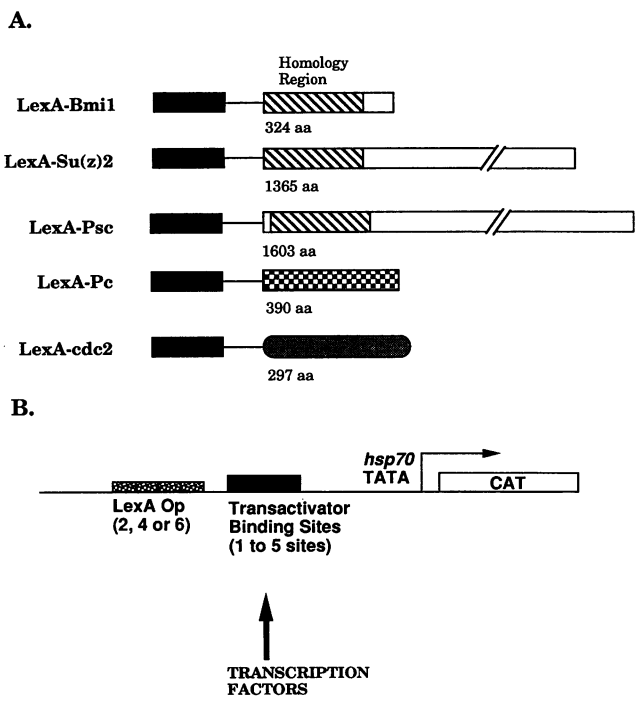


FIG. 1. Schematic of LexA fusion proteins and reporter constructs. (A) Full-length LexA (aa 1 to 202) was fused to the influenza virus hemagglutinin epitope tag followed by the indicated test protein. The Bmi-1 and Pc fusions contain the respective full-length proteins; the Su(z)2 fusion contains aa 2 to 1365, and that of Psc contains aa 12 to 1603. The number of amino acids given beneath each schematic fusion is the full length of the protein used in the fusion to LexA. The cross-hatched regions of Bmi-1, Su(z)2, and Psc correspond to the 200-aa homology domain, which contains the pair of putative zinc fingers (17, 48). The encoding genes are all expressed from the cytomegalovirus immediate-early promoter on the vector CDM8 and have SV40 splice and polyadenylation signals at the 3′ ends. (B) Reporter plasmids used to test repression by LexA fusion proteins contain the CAT gene with the human *hsp70* TATA region (−40 to +160) and, immediately upstream, binding sites for different transactivators. Operators (Op) for LexA were placed distal to activator binding sites. Operators were positioned 10 to 20 bp upstream, except where indicated (see Fig. 6).

RESULTS

Pc-G proteins repress activator function. We initially set out to characterize the function of Bmi-1, a candidate mammalian Pc-G protein, in mammalian cells. Upon finding that Bmi-1 functioned as a repressor (see below), we extended our analysis to two *Drosophila* proteins, Psc and Su(z)2, that have a high degree of sequence similarity to Bmi-1, and we also analyzed the Pc protein itself. While the *Su(z)2* gene does not have a Pc-G phenotype, it is part of a gene locus with the *Psc* gene, is highly related to *Psc*, and affects the mutant phenotype caused by the *Psc* gene (1).

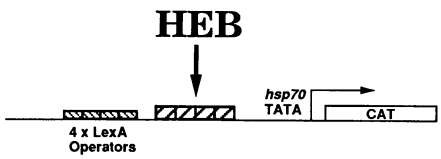
We targeted Bmi-1, Psc, Su(z)2, and Pc to a specific site by fusing the coding regions of these genes to the bacterial LexA protein (Fig. 1A). To analyze the functions of these fusion proteins, we constructed CAT reporter genes that contained the human *hsp70* TATA region, binding sites for various activator proteins immediately adjacent to the TATA region, and LexA binding sites distal to the activator binding sites (Fig. 1B). We then introduced the LexA fusion construct, the reporter gene, and any required activator into BALB/c 3T3 cells and determined the effect of each LexA fusion on activator function.

Figure 2 shows the results of such an experiment when the activator is either the transfected HEB protein (a basic region–helix-loop-helix factor) (19) or the endogenous HSF. An hGH-expressing construct, included in all transfections, was used to control for transfection efficiency (see Materials and Methods). Transfected HEB was responsible for essentially all of the activity of the HEB reporter construct (compare lines 1 and 2 in Fig. 2), and heat-induced, endogenous HSF mediated most of the activity of the HSF reporter (compare lines 15 and 16). Introducing LexA–Bmi-1, LexA-Su(z)2, or LexA-Pc repressed activation conferred by either HEB (compare line 2 with lines 4 to 6) or endogenous HSF (compare line 16 with lines 18 to 20). LexA-Su(z)2 was a more potent repressor than either LexA–Bmi-1 or LexA-Pc and repressed expression as much as 30-fold. LexA alone and LexA-cdc2 did not repress expression significantly, and repression was not seen when Bmi-1, Su(z)2, or Pc was expressed without the LexA DNA binding domain (lines 7 to 10 and 21 to 24). Finally, LexA binding sites were required to observe repression of the reporter constructs by the LexA fusion proteins (compare line 11 with lines 12 to 14 and line 25 with lines 26 to 28). We conclude that Bmi-1, Su(z)2, and Pc can function as repressors when they are targeted to a promoter on transiently introduced DNA.

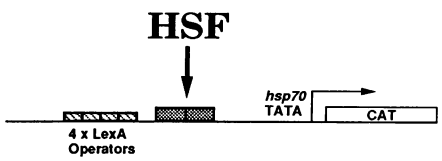
Differences in the ability to repress different activation domains. In the experiments reported above, and in other experiments with other activators (data not shown), we noticed that the ability of any of the LexA fusions to repress was frequently different with different activators. For example, LexA-Pc consistently repressed HSF more effectively than HEB (12-fold versus 3.5-fold; Fig. 2). This might be caused by a differential effect on the ability of different activator proteins to bind to the reporter construct or might be caused by a differential ability of the fusions to repress the function of different activation domains. To distinguish between these possibilities, we analyzed the ability of the LexA fusion proteins to repress GAL4 fusions that contained various activation domains. If the LexA fusions function primarily by directly interfering with activator binding, then we would anticipate that all of the GAL4 fusions would be repressed similarly by a given fusion. If instead the LexA fusions repress activation domain function, we would anticipate differential repression of the various GAL4 fusions.

The relative activities of five GAL4 fusions containing heterologous activators and amino acids 1 to 147 of GAL4 [GAL4(1–147)], which contains a weak activation domain, were measured by using reporter constructs with two GAL4 sites (Fig. 3A). The strengths of these activators vary over 100-fold in our assay system. Three activation domains that contain acidic residues (HSF1, VP16, and the artificial amphipathic helix [AH]) were the most potent activators, while the Jun and Sp1 activation domains were considerably less potent. The repressor activities of LexA-Su(z)2, LexA-Psc, LexA-Pc, and LexA–Bmi-1 varied as the activation domain was changed (Fig. 3B and C; note that the scales on the fold repression axes differ in these two panels). This was most noticeable with LexA-Su(z)2 and LexA-Psc. For example, LexA-Su(z)2 repressed GAL4-VP16 and GAL4 (1–147) 10-fold but repressed GAL4-AH and GAL4-Jun 25-fold. LexA-Psc repressed both GAL4-HSF1 and GAL4-VP16 over 20-fold but repressed GAL4-Sp1 and GAL4 (1–147) less than 5-fold. LexA–Bmi-1 and LexA-Pc showed differential repression as well, although over a more narrow range. Interestingly, each LexA fusion showed a different pattern of repression of these GAL4 fusions. These data demonstrate that the effect of a LexA

A.



B.



	Trans Factors	CAT Activity (Normalized)	Fold Repression Single Exp't	Fold Repression Average
4x LexA Sites:				
1.	None	1.0		
2.	HEB Alone	1900	-	
3.	HEB + LexA	1700	1.1	1.1+/-0.35
4.	HEB + Lex-Bmi1	520	3.7	3.9+/-0.94
5.	HEB + Lex-Su(z)2	40	48	30+/-9.2
6.	HEB + Lex-Pc	610	3.1	3.5+/-0.36
7.	HEB + Lex-cdc2	1200	1.6	1.6+/-0.15
8.	HEB + Bmi1	7600	0.25	0.36+/-0.15
9.	HEB + Su(z)2	1100	1.7	1.2+/-0.43
10.	HEB + Pc	1300	1.5	1.2+/-0.38
No LexA Sites:				
11.	HEB Alone	1500	-	
12.	HEB + Lex-Bmi1	1800	0.83	0.77+/-0.19
13.	HEB + Lex-Su(z)2	2200	0.68	0.78+/-0.09
14.	HEB + Lex-Pc	1400	1.1	0.98+/-0.51

	Trans Factors	CAT Activity (Normalized)	Fold Repression Single Exp't	Fold Repression Average
4x LexA Sites:				
15.	None	54		
16.	HSF Alone	600	-	
17.	HSF + LexA	490	1.2	1.0+/-0.19
18.	HSF + Lex-Bmi1	140	4.3	4.3+/-0.53
19.	HSF + Lex-Su(z)2	25	24	15+/-5.9
20.	HSF + Lex-Pc	130	4.6	12+/-5.0
21.	HSF + Lex-cdc2	380	1.6	1.3+/-0.34
22.	HSF + Bmi1	510	1.2	1.1+/-0.25
23.	HSF + Su(z)2	370	1.6	0.93+/-0.23
24.	HSF + Pc	630	1.2	2.0+/-1.9
No LexA Sites:				
25.	HSF Alone	490	-	
26.	HSF + Lex-Bmi1	1700	0.29	1.1+/-1.0
27.	HSF + Lex-Su(z)2	990	0.49	2.4+/-2.1
28.	HSF + Lex-Pc	840	0.58	1.5+/-1.1

FIG. 2. Repression of HEB- and HSF-mediated expression by LexA fusion proteins. (A) Repression of activation by transfected HEB. The CDM8HEB expression vector (4.0 μg) (19) was cotransfected into BALB/c 3T3 cells with the X523hspCAT reporter plasmid (0.5 μg), which contains four LexA operators immediately 5′ of four copies of an array consisting of μE5, μE2, and μE3 from the immunoglobulin heavy-chain enhancer and the *hsp70* TATA, along with a CDMLex- or CDMf-based expression vector (4.0 μg) encoding the protein indicated on the left of panel A (see Materials and Methods for details). Reporter expression was undetectable in the absence of HEB. The controls with a reporter lacking LexA operators were done with 523CAT, the parental reporter plasmid (38). (B) Repression of activation by endogenous HSF. HSF is induced to activate transcription upon heat shock. BALB/c 3T3 cells were cotransfected with the XSHSECAT reporter (0.5 μg) containing an optimized heat shock element and a vector (4.0 μg) expressing the protein indicated on the left of panel B. HSF was activated in transfected cells by a 1-h heat shock at 43°C at 48 h posttransfection, followed by a 6-h recovery at 37°C. Control experiments with a reporter lacking LexA operators were done with SHSECAT. Numbers given for normalized CAT activity represent the average activity obtained from duplicate transfections, which has been normalized for transfection efficiency. Normalization was to hGH expressed from cotransfected pXGH5 (Nichols Diagnostic). Identical results were obtained when LacZ was used for normalization (data not shown). The effect of cotransfecting the different expression vectors encoding the test proteins is presented as fold repression, i.e., the ratio of normalized CAT activity with activator alone divided by that in the presence of the given expression construct. Data generated from a single representative experiment are presented in columns for CAT activity (normalized) and fold repression, single experiment, for comparison with the average fold repression. Average repression and standard deviation were determined from at least four independent experiments.

fusion on a particular activator is determined at least in part by the activation domain.

The LexA fusions had a repressive effect on expression from the reporter containing two GAL4 sites in the absence of any added GAL4 activator (data not shown). It could be argued, therefore, that these LexA fusions were able to inhibit both basal transcription, i.e., that which is mediated by an initiation complex alone, and activated transcription. This point cannot be rigorously determined from these experiments, however, as the very low level of expression from the promoter in the absence of GAL4 [more than 10-fold lower than the activity seen with GAL4(1–147)] might be regulated by endogenous activators that bind fortuitously to sites on the transfected reporter DNA.

We were concerned that certain of the activators might be saturating for activity on the reporter constructs used above, as this might influence the degree of repression observed. We compared the activities of the activators on a reporter containing five GAL4 sites, as opposed to the two-site reporter used above. Activation of the reporter gene was more efficient with five sites than with two sites with each of the GAL4 activators, demonstrating that activation of the reporter is not saturating under the conditions used to test our repressor fusions (data not shown).

A.

Gal4 Fusion Activation Domain:	HSF1	VP16	AH	Jun	Sp1	Gal1-147
Relative Strength:	260	67	8.8	2.7	1.9	1.0

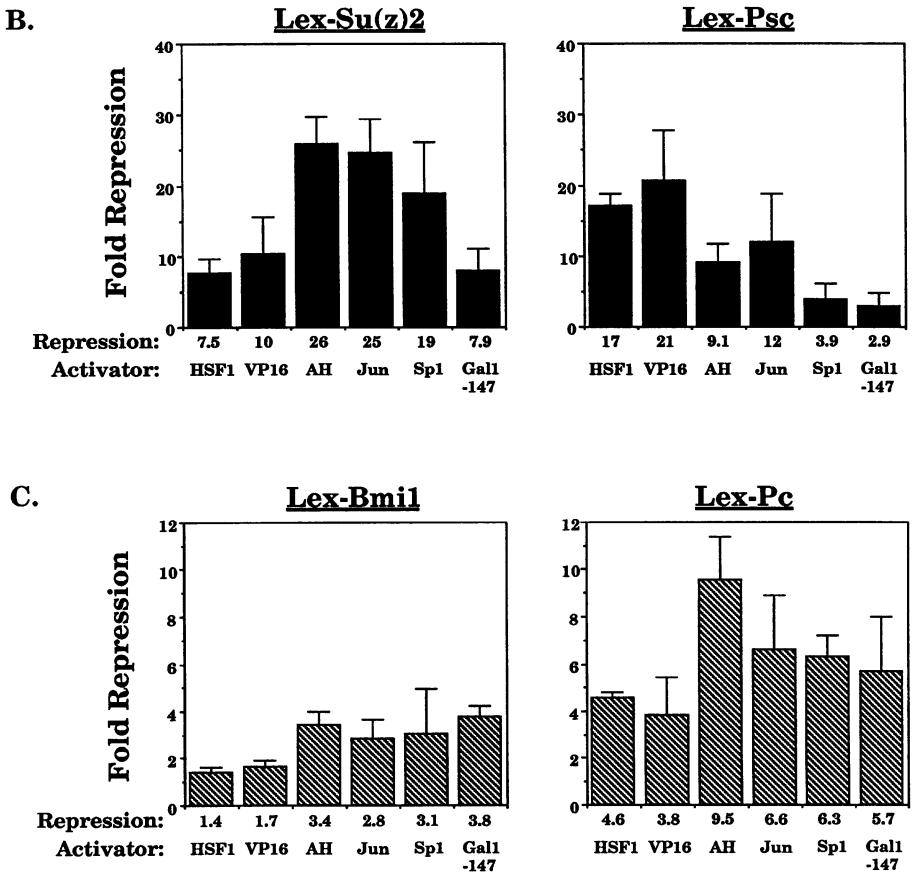


FIG. 3. Differential repression by each LexA fusion of different GAL4 activators. (A) Relative strengths of GAL4 fusion proteins containing the indicated activation domain. The relative strengths were determined in parallel cotransfections with each activator and the reporter plasmid, X4G2CAT, used in the experiments presented. Activity numbers were normalized for transfection efficiency (hGH) and the amount of lysate assayed; the time of the assays was kept constant. GAL4(1–147) was the weakest activator and was arbitrarily defined as 1.0 strength unit. The strengths of the other activators are relative to that of GAL4(1–147). The reporter X4G2CAT contains four LexA operators approximately 20 bp 5′ of two GAL4 sites and the *hsp70* TATA element. (B) Lex-Su(z)2 and Lex-Psc were potent repressors. Lex-Su(z)2 and Lex-Psc reduced expression of the target reporter plasmid up to 20- to 30-fold with different GAL4 fusion activators. (C) Lex-Pc showed moderate repression of each activator, while Lex–Bmi-1 was a weaker repressor. Repression by Lex-Pc was between 4- and 10-fold, and Lex–Bmi-1 repressed different GAL4 fusion activators from 2- to 4-fold. Bars representing repression are arranged in order of decreasing activator strength (see panel A). Fold repression represents the ratio of CAT expression mediated by a given GAL4 activator divided by that in the presence of a LexA repressor. Repression and standard deviation bars are derived from at least four experiments, except for the GAL4(1–147) data, which were from three experiments.

We also considered the possibility that repression of activator function was caused in part by an effect of the LexA fusions on activator expression. To test this, we metabolically labeled transfected COS cells and measured by IP the amount of activator protein in the presence and absence of a LexA fusion. For example, there was no effect of LexA-Psc on GAL4-VP16 expression, nor was there an effect of LexA-Su(z)2 on GAL4(1–147) expression (Fig. 4). We conclude that the LexA fusion proteins do not inhibit activator expression, as anticipated because repression requires a LexA site and there are no LexA sites on the expression vectors for the GAL4 fusions.

We verified that the repression that we measured according to CAT activity was a reflection of the levels of appropriately initiated transcript from the reporter gene. Both primer extension assays and S1 nuclease assays demonstrated that appropriately initiated transcripts were reduced by LexA fusions to

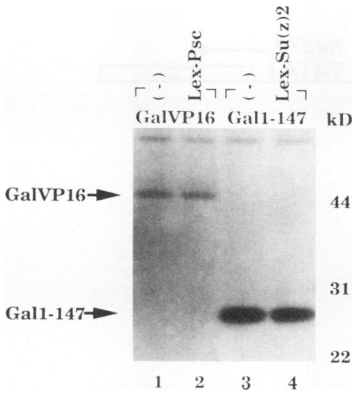


FIG. 4. LexA fusions do not alter the expression level of GAL4 activators. COS cells were transfected with GAL4 expression vectors plus and minus LexA fusion expression vectors, and whole cell lysates were prepared following metabolic labeling with [^{35}S]methionine and [^{35}S]cysteine. IPs were performed with polyclonal antisera raised against GAL4 (kindly provided by M. Ptashne); equal numbers of trichloroacetic acid-precipitable counts were used in each IP reaction. The IP complexes were separated on an SDS–10% polyacrylamide gel. Lanes 1 and 2, respectively, show IPs from cells transfected with pBXG-VP16, which expresses GAL4-VP16 (upper arrow), with and without cotransfection of CDMLex-Psc. Lanes 3 and 4, respectively, show IPs from cells transfected with pBXG1, which expresses GAL4(1–147) (lower arrow), with and without cotransfection of CMDLex-Su(z)2. Molecular size markers were run in an adjacent lane, and their positions are indicated on the right.

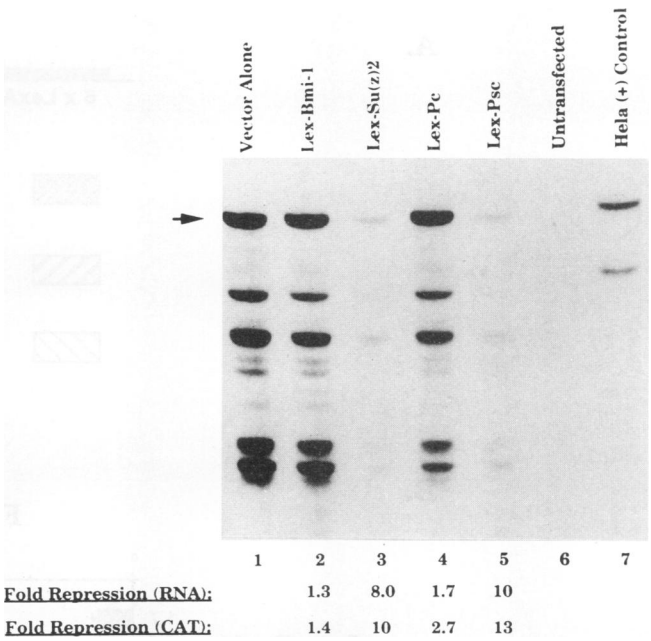


FIG. 5. LexA fusions reduce the level of CAT transcript. Lanes: 1, CAT primer extension products corresponding to transcripts made in the presence of GAL4-VP16 and in the absence of any LexA fusion; 2 to 5, levels of primer extension products made from CAT message in cells transfected with the indicated LexA fusion repressor; 6, negative control in which the RNA used for primer extension was from untransfected cells; 7, positive control for transcription start site selection, using RNA from heat-shocked HeLa cells transfected with a reporter containing the intact *hsp70* promoter. There is a few-nucleotide difference in start site for this promoter in HeLa cells versus BALB/c 3T3 cells (16). Fold repression by each LexA fusion based on CAT RNA level is on the first line below each lane and is relative to the message level in lane 1. Quantitation was done on a PhosphorImager (Molecular Dynamics). The values given correspond to the average repression based on quantitation of three different bands on the gel; faster-migrating species arose presumably from premature termination during the reverse transcription reaction or different mRNA start sites. Repression values derived from normalized CAT activity are given on the second line at the bottom of each lane. BALB/c 3T3 cells were cotransfected with the X4G2CAT reporter, a GAL4-VP16 expression vector, and the indicated LexA fusion protein expression vector. Cells harvested for CAT assays were transfected in parallel to those harvested for RNA. CAT message level was measured by primer extension with a primer specific to the 5′ end of the CAT gene. Fifty milligrams or less of total RNA (amounts varied to account for differences in transfection efficiency as measured by hGH) from transfected cells was used for each sample. Primer extension products were separated on a 5% denaturing polyacrylamide gel. Extension product length was determined from the mobility of size standards separated on the same gel. The appropriate length of the extension product is 238 nucleotides (arrow).

an extent similar to that seen in the CAT assays (Fig. 5 and data not shown).

Pc-G proteins repress from a distance. It appears that Pc-G proteins can exert their repressive effects over several kilobases in *D. melanogaster* (6, 43). We therefore analyzed the effect of changing the distance between the LexA sites and the activator site on repression by the LexA fusions. Although the level of repression mediated by these fusions decreased as their binding sites were moved away from those of the activator, all of the tested fusions actively repressed transcription from 300 bp away (Fig. 6); for example, both LexA-Su(z)2 and LexA-Psc repressed activation by GAL4-VP16 approximately 6-fold from 300 bp away, in comparison with 10- and 21-fold, respectively, when each LexA fusion was bound adjacent to GAL4-VP16. LexA-Su(z)2 showed the greatest level of repression from a distance of 300 bp, repressing activation by GAL4-AH 19-fold. LexA-Su(z)2, LexA-Psc, and LexA-Pc were active, but to a lesser extent, when bound 1,700 bp away; the maximum repression observed from this distance was fourfold (Fig. 6). We conclude that these Pc-G fusions can repress activators that are significantly separated from the site where the fusion protein is bound.

Mutant Pc-G proteins show altered repression activity. We were interested in determining whether mutations in Pc-G genes which result in homeotic transformation have an effect on the repressor activity of Pc-G proteins fused to LexA. The *Pc* gene has been extensively characterized with respect to the molecular lesions responsible for the *Pc* mutant phenotype; missense mutations and small deletions have been found clustered in the chromo domain and at the C terminus of the protein (28, 30). We tested the function of mutant Pc proteins as fusions to LexA in cotransfection assays. We examined two chromo domain mutants: Pc^{XL5}, which is a natural allele in which aa 69 and 70 were deleted, and Pc^{LO35}, which was created by deleting aa 42 to 65. These mutations were introduced into the LexA constructs by using cDNAs kindly provided by R. Paro. We generated a LexA-Pc derivative containing a C-terminal truncation of 86 aa (ΔC86) as well; this mutation is similar to those found in the alleles Pc^{2} and $Pc^{23937.17B}$, which are truncated 72 and 90 aa, respectively (30). Two more extensive C-terminal deletions of 118 aa (ΔC118) and 310 aa (ΔC310), for which no comparable natural alleles have been isolated, were tested as well.

The LexA-Pc derivative ΔC86 and those containing chromo domain mutants, LexA-Pc^{XL5} and LexA-Pc^{LO35}, had dramatically different repressor activities. Whereas wild-type LexA-Pc

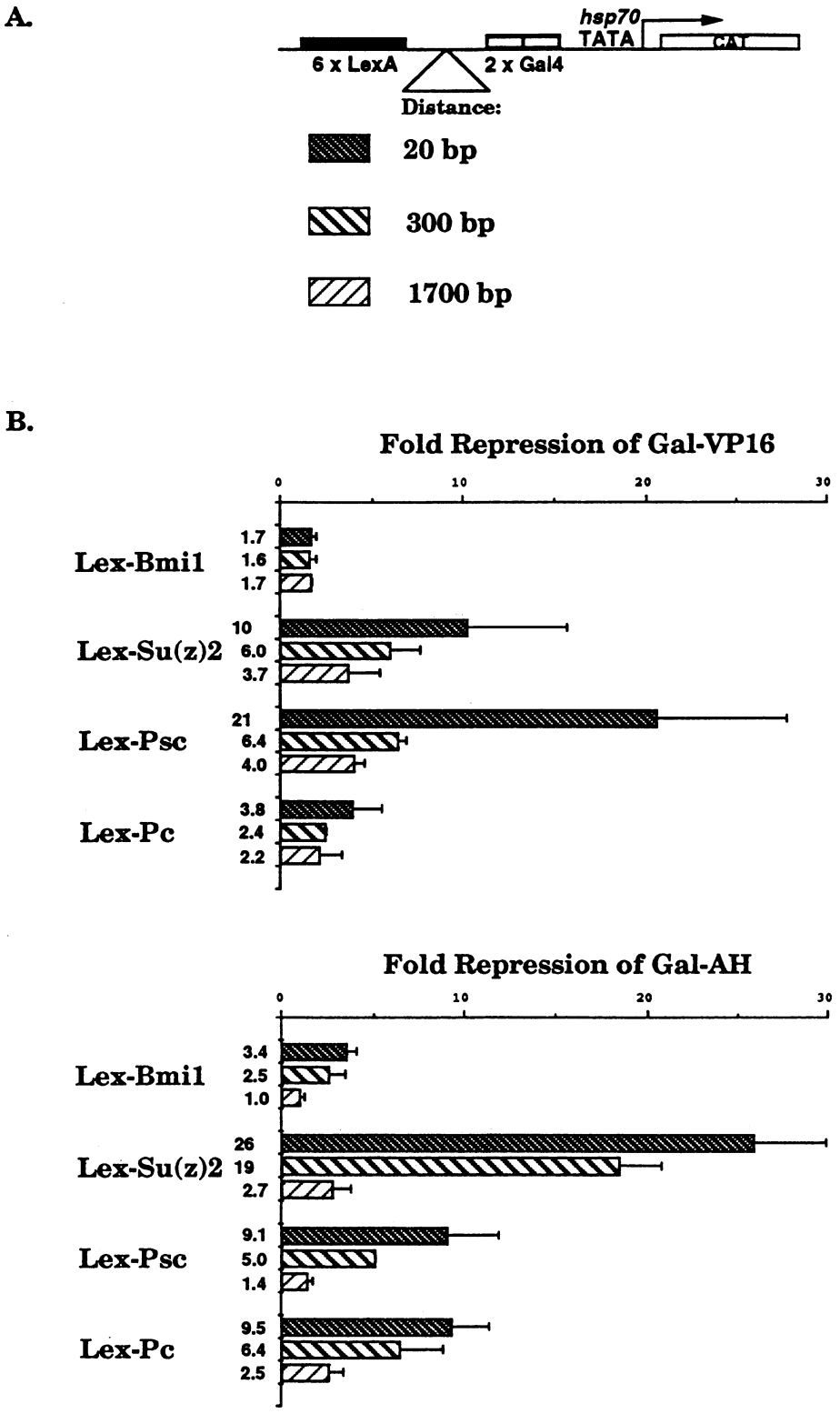


FIG. 6. LexA fusions can repress expression from a distance. (A) Schematic of reporter constructs based on X6G2CAT. The distance separating LexA operators and GAL4 sites (20, 300, or 1,700 bp) is indicated along with the corresponding pattern code used in the bar graphs in panel B. (B) Repression of GAL4-VP16 and GAL4-AH from a distance by LexA fusions. Average repression with standard deviation from at least three independent experiments is shown graphically, and the numeric value for repression is given at the left. Fold repression was determined as in Fig. 2 and 3. Transfections were identical to those described in Fig. 3, except that reporter constructs have six LexA operators; repression with proximal LexA sites was essentially the same with two, four, or six operators (data not shown).

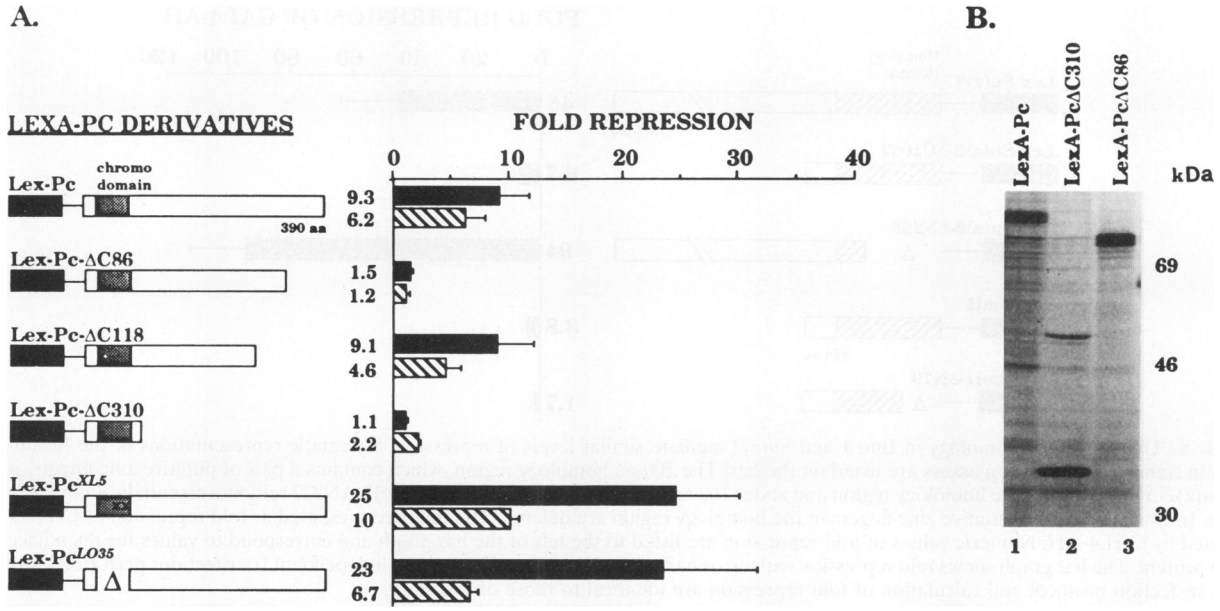


FIG. 7. Mutations in Pc alter the repressor activity of LexA-Pc. (A) The repressor activity of each LexA fusion was determined in transient transfection assays as described for Fig. 2 and 6. Repression is presented as the fold reduction in CAT reporter activity in the presence of the indicated LexA fusion relative to that in the presence of the empty expression vector. The dark stippled bars represent data from transfections in which GAL4-AH was the activator and the reporter was X4G2CAT (see Fig. 3). The striped bars represent data from transfections in which GAL4-Sp1 was the activator and the reporter was X6-300-G2CAT, in which the LexA operators are positioned 300 bp upstream of the GAL4 binding sites. Standard deviations are shown graphically, and the values for fold repression are listed to the left of the graph. All data for the different Pc mutants were generated from transfections in which wild-type LexA-Pc was tested in parallel; the fold repression given for LexA-Pc is not identical to that given in Fig. 3 and 6, since these data are from an independent set of transfections. Each LexA-Pc mutant derivative was tested in three to six separate transfections. Schematic illustrations of the LexA fusions containing full-length Pc (390 aa) and the different mutant Pc proteins are shown to the left of the graph. ΔC86, ΔC118, and ΔC310 are C-terminal truncations of Pc of the indicated number of amino acids; *XL5* and *LO35* are deletions of residues 69 to 70 and 42 to 65, respectively, in the chromo domain (28). The LexA domain is in black, and the chromo domain is cross-hatched. (B) The expression level of the inactive LexA-Pc mutants is similar to that of the wild type. IPs were performed with polyclonal antisera raised against LexA protein (kindly provided by R. Brent) and whole cell lysates from COS cells transfected with the indicated LexA-Pc derivative, labeled and harvested as in Fig. 4. IP complexes were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiographed. LexA-Pc has a predicted mass of 67 kDa, but its mobility is significantly reduced on SDS-PAGE; LexA-Pc-ΔC86 has greater mobility, as anticipated, and LexA-Pc-ΔC310 has a predicted mass of 33 kDa and runs accordingly. The nature of the species at approximately 50 kDa is unknown.

repressed activation by GAL4-AH 9.3-fold when bound 20 bp upstream on the reporter X4G2CAT, LexA-Pc-ΔC86 mediated only 1.5-fold repression of GAL4-AH; in contrast, the chromo domain mutants, *XL5* and *LO35*, mediated 25- and 23-fold repression, respectively (Fig. 7A). We also tested these LexA-Pc derivatives in cotransfections with GAL4-Sp1 and the reporter X6-300-G2CAT, in which the LexA operators are 300 bp upstream of the GAL4 sites. LexA-PC-Δ86 also demonstrated no ability to repress in this assay, while the two chromo domain mutants repressed to a degree similar to that of the fusion to wild-type Pc (Fig. 7A).

The two more extensive C-terminal deletions, ΔC118 and ΔC310, were tested against GAL4-AH and GAL4-Sp1 as described above. While Lex-Pc-ΔC310, which is truncated just C terminally of the chromo domain, showed little activity (1.1-fold repression versus GAL4-AH and 2.2-fold repression versus GAL4-Sp1), surprisingly, the repression activity of Lex-Pc-ΔC118 was greater than that of Lex-Pc-Δ C86 (9.1-fold repression versus GAL4-AH and 4.6-fold repression versus GAL4-Sp1) (Fig. 7A). Both Pc mutants (ΔC86 and ΔC310) that were defective in repression were expressed at levels similar to those of the wild-type Pc fusion (Fig. 7B).

We conclude that a C-terminal truncation (ΔC86) that mimics naturally occurring mutant *Pc* alleles also reduced repression activity in the cotransfection assay. This does not appear to be due to deletion of a repression domain in Pc, as a more extensive deletion (ΔC118) regained the ability to repress. It is possible that the incongruous results obtained with ΔC86 and ΔC118 indicate an effect of the ΔC86 mutant on the overall structure of the protein that renders it inactive. The chromo domain mutants repressed equally to or to a greater degree than wild-type Pc when fused to LexA; this finding is consistent with the hypothesis that the chromo domain is involved in localization (28). Removal of the chromo domain might lead to less competition between localization by LexA to the target reporter and endogenous sites to which the chromo domain would otherwise target the LexA-Pc fusion protein.

We were intrigued by the observation that the *Drosophila* factors repressed to a greater extent than Bmi-1 and therefore tested the role of the region of homology shared by Bmi-1, Su(z)2, and Psc in repressor function. We used deletion analysis to directly compare the repression mediated by the conserved region of Su(z)2 with that of Bmi-1. A LexA fusion to the N-terminal 298 aa of Su(z)2 alone (ΔC1067), which contains the 200-aa homology region, could repress expression by GAL4-AH nearly sevenfold (Fig. 8). This was similar to the level of repression mediated by LexA–Bmi-1 (3.8-fold) and drastically reduced in comparison with that mediated by

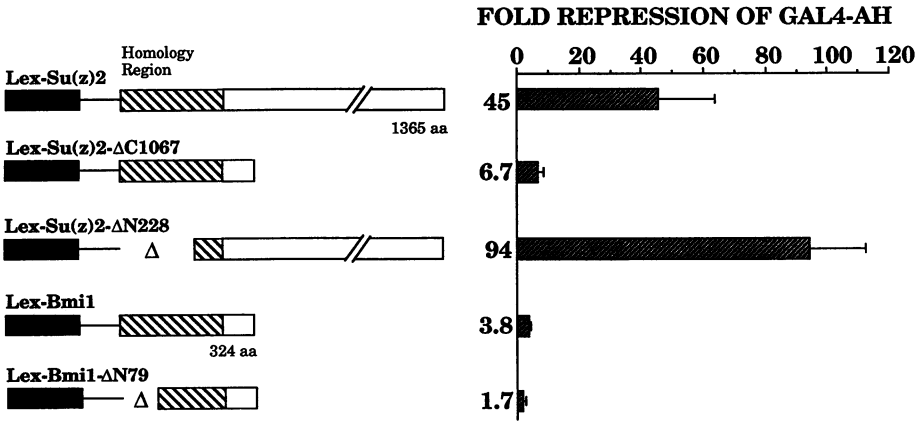


FIG. 8. The regions of homology in Bmi-1 and Su(z)2 mediate similar levels of repression. Schematic representations of the fusion proteins tested in transient transfection assays are listed on the left. The 200-aa homology region, which contains a pair of putative zinc fingers, is striped. Lex-Su(z)2-ΔC1067 leaves the homology region and about 100 aa C terminal of it, and LexA-Su(z)2-ΔN227 removes essentially all of the homology region. In Bmi1-ΔN79, the putative zinc fingers in the homology region are deleted. The data are presented as fold repression of CAT expression mediated by GAL4-AH. Numeric values of fold repression are listed to the left of the bar graph and correspond to values for the adjacent LexA fusion protein. The bar graph shows fold repression with error bars generated from at least three independent transfections performed in duplicate. The transfection protocol and calculation of fold repression are identical to those of Fig. 3.

wild-type LexA-Su(z)2 (45-fold repression). Although the conserved domain of Su(z)2 did have repressor activity, this region was unnecessary for repression mediated by the C-terminal regions of Su(z)2; in fact, removal of the homology region (ΔN227) generated a repressor that was somewhat more active than the wild-type fusion (Fig. 8). The homology region of Su(z)2 contains a pair of putative zinc fingers which may function in DNA binding; therefore, removal of this domain might enable the LexA fusion to be more efficiently targeted to the test promoter by the LexA DNA binding domain. In contrast to the case of Su(z)2, deletion of the putative zinc fingers in Bmi-1 (ΔN79) lowered repressor activity from 3.8-fold (wild type) to only 1.7-fold (Fig. 8). These deletion analyses suggest that the potent repressive effects of Su(z)2 require the C-terminal regions of the molecule that are not found in Bmi-1.

DISCUSSION

Maintaining homeotic genes in a stably repressed state is essential for appropriate development in *D. melanogaster*, and such mechanisms are likely to be important generally in eukaryotes. We demonstrate above that individual Pc-G proteins function as potent repressors in mammalian cells on promoters that have been transiently introduced. The simplest interpretation of the data is that these proteins inhibit the ability of transcriptional activation domains to function, since the ability of a Pc-G protein to repress an activator changes as the activation domain is changed (Fig. 3). This interpretation is entirely consistent with the substantial body of genetic data regarding the function of the Pc-G, and models in which the Pc-G functions to directly affect activator function have been proposed previously (20, 21). Although our data and those of others are consistent with such a model, the predominant current hypothesis concerning Pc-G function proposes that Pc-G proteins repress by inducing the formation of heterochromatin; this model has been favored primarily on the basis of the supposition that heterochromatin would be well suited to the stable maintenance function of the Pc-G.

We have not examined the effects of Pc-G proteins on chromatin structure in these experiments, and there are no data that examine these effects in other systems. While we favor the simple interpretation of direct interaction between the Pc-G proteins and the transcription machinery, it is equally plausible that tethering of Pc-G proteins to the transient reporter DNA alters local chromatin structure and thereby represses reporter expression. The observed variable repression mediated by the Pc-G proteins would thus reflect differences in the ability of activators to contend with chromatin structure.

Our observations provide a basis for further characterization of the mechanism of repression, as they point to a number of possible mechanisms which can be addressed by using the LexA fusion proteins described in this report. The Pc-G proteins could either physically or enzymatically impair the ability of the activation domain to function or could impair the ability of the components of the initiation complex to receive a signal from the activator. Experiments using in vitro systems will be required to examine these two possible mechanisms. Another mechanism by which these LexA fusions might repress is by sterically or competitively interfering with activator binding. This is not likely to be the case, since different activators based on the same GAL4 DNA binding domain were differentially repressed by a given Pc-G protein (Fig. 3). In addition, repression could occur from 300 and 1,700 bp away (Fig. 6), observations that are difficult to reconcile with steric interference of binding. Finally, Pc-G proteins could interfere with binding of the activators by an effect on local chromatin structure. The differential effect observed as the activation domains are changed might then be explained by a differential ability of activation domains to promote DNA binding by the activator in chromatin.

It is striking that the *Drosophila* Pc-G proteins function as repressors in mammalian cells. This finding suggests that the mechanism of repression is conserved between mammals and *D. melanogaster*. This observation, in combination with the discovery of mammalian genes that are related to Pc-G genes (5, 33, 47), argues that similar repressive functions are important in the regulation of mammalian genes. The products of the oncogene *bmi-1*, a mammalian homolog of *Psc* and *Su(z)2*, had repressor activity but was less effective than the *Drosophila* proteins. The Bmi-1 protein is significantly smaller than its

Drosophila counterparts Su(z)2 and Psc (Fig. 1), and the corresponding homology region of Su(z)2 alone when fused to LexA exhibited a level of repressor activity similar to that of Bmi-1 (Fig. 8). The increased repressive effects of Su(z)2 appear to be mediated by the C-terminal regions of the molecule that are not found in Bmi-1. Therefore, although Bmi-1, Psc, and Su(z)2 show sequence homology and appear to have similar functions, there may be other mammalian proteins with homology to Su(z)2 and Psc that function as more potent repressors, analogous to the *Drosophila* proteins.

We attempted to correlate the effects of naturally occurring mutations in Pc-G genes with the repressive effects observed here. We examined two mutations in the chromo domain of *Pc* (Pc^{XL5} and Pc^{LO35}). Introduction of these mutations into LexA-Pc did not impair repressor activity and, in fact, increased repression by more than twofold (Fig. 7). These mutants appear to affect targeting of Pc (28), so it was not unexpected that the repressor activity of LexA-Pc, which was targeted by the LexA domain, was not affected by these mutations. Other *Pc* alleles contain C-terminal deletions (30). Deletion of 86 aa in the C terminus of *Pc* (similar to naturally occurring deletions) eliminated the ability of the LexA-Pc fusion to repress (Fig. 7A), offering a correlation between repressive effects in our transfection assay and effects on *Drosophila* development. This mutant was expressed at the same level as the normal LexA-Pc fusion (Fig. 7B). Confounding a strong interpretation of these results, however, was the observation that a LexA-Pc fusion with a more extensive deletions in the C terminus (removing 118 aa) regained the ability to repress. It should be noted that this deletion is more extensive than that in any of the characterized *Pc* alleles.

We conclude that Pc-G proteins are able to dramatically repress activator function on transiently introduced DNA and that these repressors can function, albeit less well, from distances up to 1,700 bp from the promoter. We argue that this effect is likely to be an important part of the ability of Pc-G proteins to maintain homeotic genes in a repressed state. We propose that the targeting of Pc-G proteins to specific loci positions them to directly inhibit activator function and thereby maintain the target promoter in a repressed state. The observation that the underlying mechanism is conserved from *D. melanogaster* to mammals argues that it is important in regulating development.

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