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The *Escherichia coli LexA* repressor-operator system works in mammalian cells

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We have demonstrated the use of the *Escherichia coli LexA* repressor-operator system to down-regulate gene expression in mouse cells. The *LexA* gene was placed downstream of the RSVLTR promoter with polyadenylation and splice signals from SV40. This expression unit was introduced into mouse L^{tk^-} cells by calcium phosphate transfection and stable transfectants selected which express LexA protein. We have used the bacterial chloramphenicol acetyltransferase gene (CAT) as our reporter gene. Transcription of this gene was driven by the HSV *tk* promoter, into which we have introduced one or two synthetic LexA operator sequences in various positions throughout the promoter. Necessary 3' signals were from the HSV *tk* gene. Repression by LexA was assessed by comparing the transient expression of tkCAT target constructs, containing LexA operator sequences in the promoter, in cells expressing LexA protein with that in control cells not expressing the repressor. We have observed up to 10-fold repression of CAT expression in $LexA^+$ cells from promoters containing LexA operator sequences.

Key words: DNA-binding protein/*Lex*A repression/*tk* promoter

Introduction

The specific interaction of proteins with DNA is ubiquitous in the control of gene expression in both eukaryotes and prokaryotes. Most progress in understanding these interactions has been made in studying bacterial repressor proteins. These proteins recognize and bind as dimers to short operator sequences (14-20 bp) in DNA, sites which have in common a property of 2-fold symmetry. Monomers of these proteins exhibit a conserved α-helical motif consisting of two α-helices that are linked by a tight turn. High resolution X-ray crystallography of the 434 and *trp* repressors bound to their respective operator sequences reveals that, at each half-site of the B-form operator DNA, the $\alpha 3$ recognition helix of each monomer lies in the major groove of the DNA (Anderson *et al.*, 1985, 1987; Schevitz *et al.*, 1985; Zhang *et al.*, 1987). The other α-helix, $\alpha 2$, lies across the groove and interacts with the phosphate groups of the DNA helix backbone.

The controlled switching of gene expression is desirable in the investigation of the function of particular genes, and is necessary if the gene product is toxic to the cells. The use of bacterial repressor-operator systems in mammalian cells could provide a useful alternative to the inducible vector systems already available which include the heat-shock protein promoters, metal ion-inducible mouse and human metallothionein promoters and the glucocorticoid-inducible mouse mammary tumour virus long terminal repeat (MMTV LTR) (Gorman, 1985). Furthermore, bacterial repressors could be engineered to recognize a naturally occurring sequence in the control region of a eukaryotic gene which would allow investigation of the effects of down-regulation of that gene in cell culture, whole animals or plants. The feasibility of achieving this goal has been demonstrated using two approaches. Firstly, Wharton *et al.* (1984) and Wharton and Ptashne (1985) have shown that it is possible to alter the recognition specificity of repressors by swapping the α-recognition helix or by site-directed mutagenesis which changes the interacting amino acids on the outside of the helix. Secondly, Youderian *et al.* (1983) and Benson *et al.* (1986) have developed bacterial selection systems for the isolation of mutant repressor proteins with new DNA sequence specificities.

Brent and Ptashne (1984) first demonstrated that the bacterial repressor LexA, similar to those described above, could repress gene expression in eukaryotes. LexA is a small repressor (202 amino acid, 22 300 daltons) which binds as a dimer to a specific 20-bp operator sequence in DNA with an apparent dissociation constant (K_d) of $< 10^{-9}$ M (Brent and Ptashne, 1984). When the operator sequence for LexA was inserted between the UAS and the TATA box of the yeast gene *GAL1*, transcription was repressed up to 10-fold in yeast cells expressing LexA protein. Insertion of the operator sequence 28 bp upstream of the UAS did not affect transcription.

We aimed to extend the work of Brent and Ptashne (1985) into mammalian cells and use the bacterial LexA protein to repress the expression of a suitable reporter gene. We have constructed a mouse cell line which expresses LexA protein and used this to demonstrate repression from a suitably engineered promoter, containing LexA operator sequences, linked to a reporter gene. Hu and Davidson (1987) and Brown *et al.* (1987) have recently demonstrated the use of another *Escherichia coli* repressor, LacI, to repress gene expression in mammalian cells and shown that repression can be alleviated using the inducer IPTG. Figge *et al.* (1988) have shown that LacI is also effective when the target gene is integrated into the chromosome.

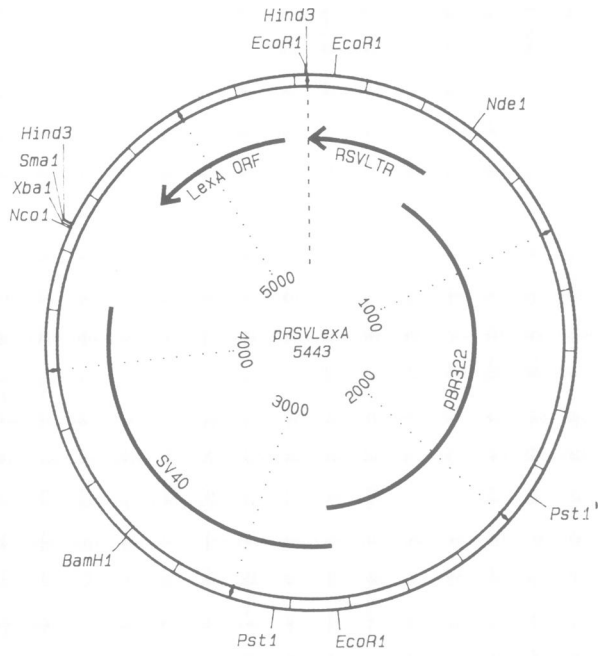

Fig. 1. The structure of the LexA expression vector pRSVLexA.

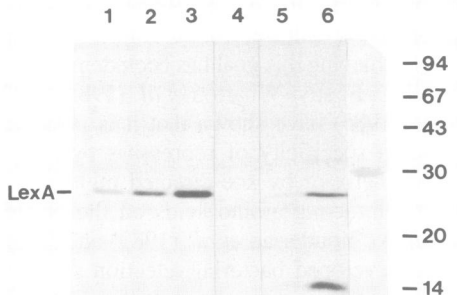

Fig. 2. A Western blot showing transient expression of LexA protein from pRSVLexA in L^{tk} cells. **Tracks 1–3**, purified LexA protein, 50, 100 and 200 ng respectively; tracks 4–6, crude cytosolic cell extracts prepared from 2×10^6 cells after transfection with carrier DNA only (**4**), 2 and 20 μg pRSVLexA DNA (**5** and **6**). The right-hand track contains mol. wt markers (kd). SDS–PAGE electroblotting and detection using anti-LexA antibody and a dye/peroxidase conjugate system are described in Materials and methods.

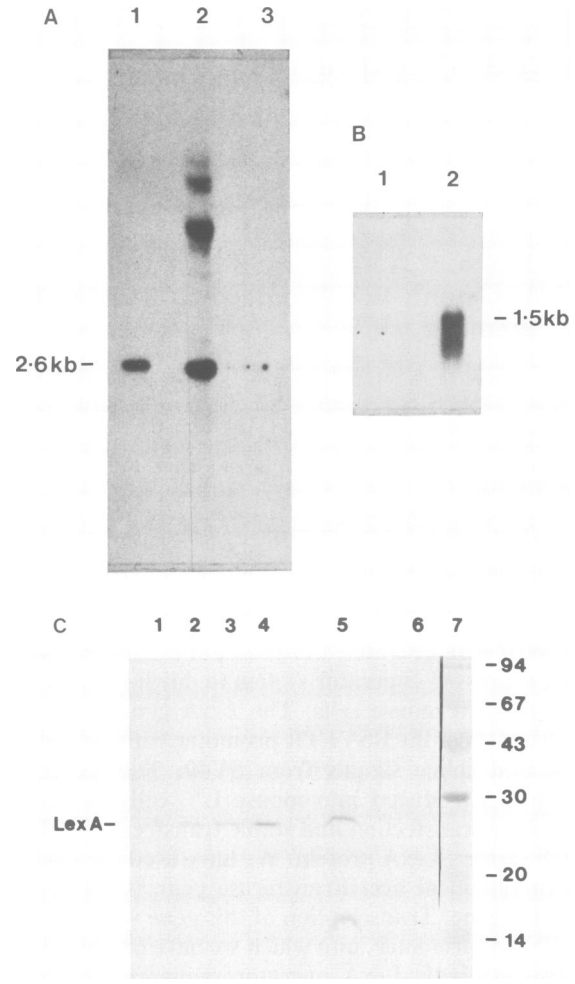

Fig. 3. Characterization of the LexA-producing cell line L^{LexA+}. (**A**) Southern blot of 5 μg chromosomal DNA from L^{LexA+} cells (**track 2**) and L^{tk-} cells (**track 3**) with 200 pg pRSVLexA DNA (equivalent to 20 copies/genome) in **track 1**. DNAs were digested with *Nde*I and *Bam*HI to release the 2.6-kb expression unit. The probe was the 900-bp *Hin*dIII fragment encoding the *Lex*A gene oligo-labelled with [^{32}P]dCTP. (**B**) Northern blot of 5 μg total RNA from L^{tk-} cells (**track 1**) and L^{LexA+} cells (**track 2**), the probe was as described for (A). (**C**) Western blot: **tracks 1–4**, purified LexA protein, 25, 50, 75 and 100 ng respectively; **tracks 5 and 6**, crude cytosolic cell extracts prepared from 2×10^6 L^{LexA+} and L^{tk-} cells respectively. **Track 7** contains mol. wt markers (kd).

Results

Expression of LexA repressor protein in mouse cells

We have constructed a LexA expression vector, pRSVLexA (Figure 1), by replacing the CAT gene in pRSVCAT (Gorman, 1985) with LexA coding sequence. This vector was first tested for transient expression of LexA protein in mouse L^{tk-} cells. L^{tk-} cells (2×10^6) were transfected with 2 or 20 μg covalently closed circular pRSVLexA DNA or carrier DNA only. The cells were harvested 24 h after transfection and crude cytosolic cell extracts analysed, using polyacrylamide gel electrophoresis (PAGE) and Western blotting, using anti-LexA antibody. We were able to detect at least 100 ng LexA protein in the extract prepared from cells transfected with 20 μg pRSVLexA (Figure 2).

To obtain cell lines with pRSVLexA integrated into the chromosome we cotransfected L^{tk-} cells with pRSVLexA and pTK1 DNA. The plasmid pTK1 (Lang *et al.*, 1984) carries the gene for thymidine kinase and allows selection for cells which are expressing that DNA in HAT medium (Gorman, 1985). Dishes (9 cm) containing 1×10^6 cells were transfected with 25 or 50 μg of pRSVLexA plus 1 μg pTK1 DNA. The DNAs were linearized by digesting with the enzymes *Bam*HI and *Hin*dIII respectively. After 2–3 weeks in selective medium 16 colonies were picked, grown up and cell extracts screened for expression of LexA protein using Western blots. Varying levels of expression of LexA protein were detected in 10 of these clones. We chose to work with one of the clones producing the highest levels of LexA protein, CT5.1 (L^{LexA+}). Southern blotting indicated that ~50 copies of pRSVLexA had integrated into the chromosome (Figure 3A). A Northern blot of total RNA revealed transcripts of sufficient length to encode the LexA

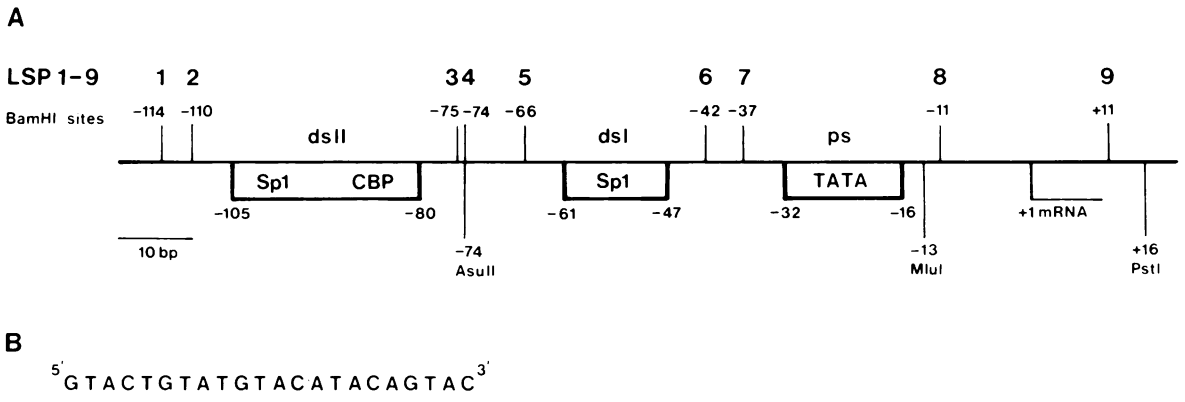

Fig. 4. (**A**) The HSV *tk* promoter. The essential regions of the promoter are boxed and represent the binding sites for the transacting factors Sp1, CAAT-binding protein (CBP) and TATA-binding protein. Ticks indicate the positions at which operator sequences were inserted, into the *Bam*HI sites of the nine different mutant LS promoters, and into three unique restriction enzyme sites within the promoter. Details of the construction of the LO-promoters containing LexA operator sequences are given in Materials and methods. (**B**) The sequence of the LexA operator used in the construction of the LO-promoters.

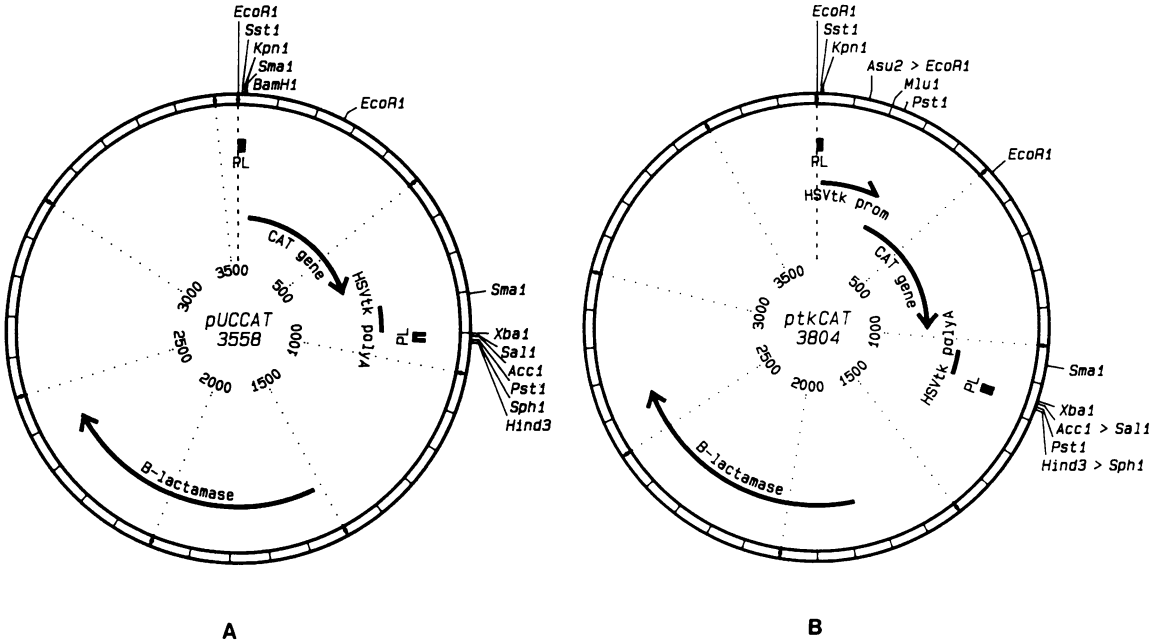

Fig. 5. (**A**) The reporter gene vector pUCCAT. (**B**) The basic reference plasmid ptkCAT which contains the wild-type *tk* promoter upstream of the CAT gene. PL indicates restriction enzyme sites originally derived from the vector pUC18.

protein (Figure 3B). Production of LexA protein was estimated, using Western blotting, by comparison with known concentrations of purified LexA protein (Figure 3C); 2×10^6 L^{LexA+} cells yielded at least 100 ng LexA protein which is equivalent to 7×10^5 dimers/cell (mol. wt LexA dimer = 44 600). Assuming the volume of the nucleus to be 10% of the total cell volume we would estimate 7×10^4 dimers LexA per nucleus.

The construction of target promoter–reporter gene fusions containing LexA operator sequences

The reporter gene we chose to test for repression by LexA encodes bacterial chloramphenicol acetyltransferase (CAT). This gene was linked to a promoter into which we could insert the LexA operator sequence, at different positions, in non-essential regions of the promoter. We have used the HSV thymidine kinase (*tk*) promoter, which has been extensively characterized (Coen *et al.*, 1986; McKnight, 1983) and the essential regions of the promoter identified by deletion analysis and insertion mutagenesis. McKnight has constructed a series of linker-scanning (LS) mutant promoters which contain a *Bam*HI site inserted at various positions along the length of the promoter. Nine of these LS mutant promoters have insertions in apparently non-essential regions of the promoter at sites that could accommodate 10–30 bp of inserted DNA without seriously impairing promoter function (McKnight, 1982). We have used these promoters (referred to as LSP1–9) for the insertion of LexA operator sequences. We have also inserted operators at three naturally occurring unique sites in the *tk* promoter (*Asu*II, *Mlu*I and *Pst*I). In total we have generated 12 different LexA operator–*tk* promoter (LO-promoter) constructs, with one or two operators inserted at each position. Figure 4A illustrates the *tk* promoter, showing the essential regions for promoter activity (boxed) and the positions of the sites used for the insertion of operator

Table I. Expression of CAT activity from the LO-promoters and their reference plasmids in L^{tk-} and L^{LexA+} cells

Plasmid	Site of operator insert[a]	Number of operators inserted	CAT activity			
			L^{tk-} cells relative activity	L^{LexA+} cells relative activity		Repression (% reduction in activity)
			[b]	[b]	[c]	
ptkCAT	–	0	100	99	99	–
ptkA1CAT	−74	1	84	73	83	17
ptkM1CAT	−13	1	107	69	64	36
ptkP1CAT	+16	1	45	21	47	53
pLSP1.0CAT	–	0	107	100	93	–
pLSP1.1CAT	−114	1	103	112	109	0
pLSP1.2CAT	−114	2	98	104	106	0
pLSP2.0CAT	–	0	102	103	101	–
pLSP2.1CAT	−110	1	103	103	100	0
pLSP2.2CAT	−110	2	100	99	99	0
pLSP3.0CAT	–	0	101	99	98	–
pLSP3.1CAT	−75	1	106	105	99	0
pLSP3.2CAT	−75	2	100	95	95	0
pLSP4.0CAT	–	0	108	110	102	–
pLSP4.1CAT	−74	1	104	89	84	16
pLSP4.2CAT	−74	2	26	19	74	26
pLSP5.0CAT	–	0	104	107	103	–
pLSP5.1CAT	−66	1	81	37	46	54
pLSP5.2CAT	−66	2	31	7	23	77
pLSP6.0CAT	–	0	104	114	109	–
pLSP6.1CAT	−42	1	99	50	50	50
pLSP6.2CAT	−42	2	59	6	10	90
pLSP7.0CAT	–	0	109	115	105	–
pLSP7.1CAT	−37	1	98	39	39	61
pLSP7.2CAT	−37	2	46	5	10	90
pLSP8.0CAT	–	0	106	104	98	–
pLSP8.1CAT	−11	1	96	70	73	27
pLSP8.2CAT	−11	2	104	54	52	48
pLSP9.0CAT	–	0	105	105	100	–
pLSP9.1CAT	+11	1	83	39	48	52

[a]The position of the inserted operator sequence(s) is indicated in base pairs relative to the transcription start point +1.
[b]CAT activity is expressed as a percentage of the reference plasmid ptkCAT in L^{tk-} cells.
[c]CAT activity for each construct is expressed as a percentage of the activity of that same construct in L^{tk-} cells, giving a direct assessment of the reduction in expression due to repression.

sequences (ticks). Figure 4B shows the sequence of the LexA operator used to construct the LO-promoters (Brent and Ptashne, 1984). The *tk* promoters were cloned into M13 vectors to facilitate insertion of operator sequences and verification by DNA sequencing. The resulting LO-promoters were placed upstream of the *E.coli* Tn*9* CAT gene with 3′ polyadenylation signals from the HSV-1 *tk* gene. Reference plasmids were constructed by placing the wild-type *tk* promoter or the mutant LS promoters without operator inserts upstream of the CAT gene. Figure 5A shows the basic CAT vector pUCCAT into which the promoter fragments were cloned. Figure 5B shows the main reference plasmid ptkCAT, constructed by inserting the wild-type *tk* promoter into pUCCAT. Details of the procedures used to generate all the constructs are described in Materials and methods.

Repression of gene expression from promoters containing LexA operator sequences in cells expressing LexA protein

The activity of the LO-promoters and their reference promoters was measured by assaying for the transient expression of the reporter gene CAT 24 h after transfection into L^{tk-} or L^{LexA+} cells. The activities of the LO-promoter−CAT plasmids were compared with those of their reference plasmids in L^{tk-} cells to check for any alteration to promoter activity which might have resulted from the insertion of operator sequences. The constructs were then used to test for repression by comparing the transient expression of CAT from each LO-promoter in L^{tk-} cells with that in L^{LexA+} cells. All the plasmids constructed are listed in Table I, indicating the position of insertion of LexA operator sequences and the number of operators inserted. The position of operator insertions can be subdivided into five regions (refer to Figure 4A): (i) upstream of all the essential elements of the promoter; (ii) between the second distal signal (dsII) and the first distal signal (dsI); (iii) between dsI and the proximal signal (ps); (iv) between ps and the transcription start point; and (v) downstream of the transcription start point.

In standard transfection experiments duplicate 9-cm dishes containing 1×10^6 L^{tk-} or L^{LexA+} cells were transfected with 10 μg of plasmid DNA, plus 10 μg carrier DNA (denatured salmon sperm DNA). The concentrations of plasmid DNA preparations used for the transfections were

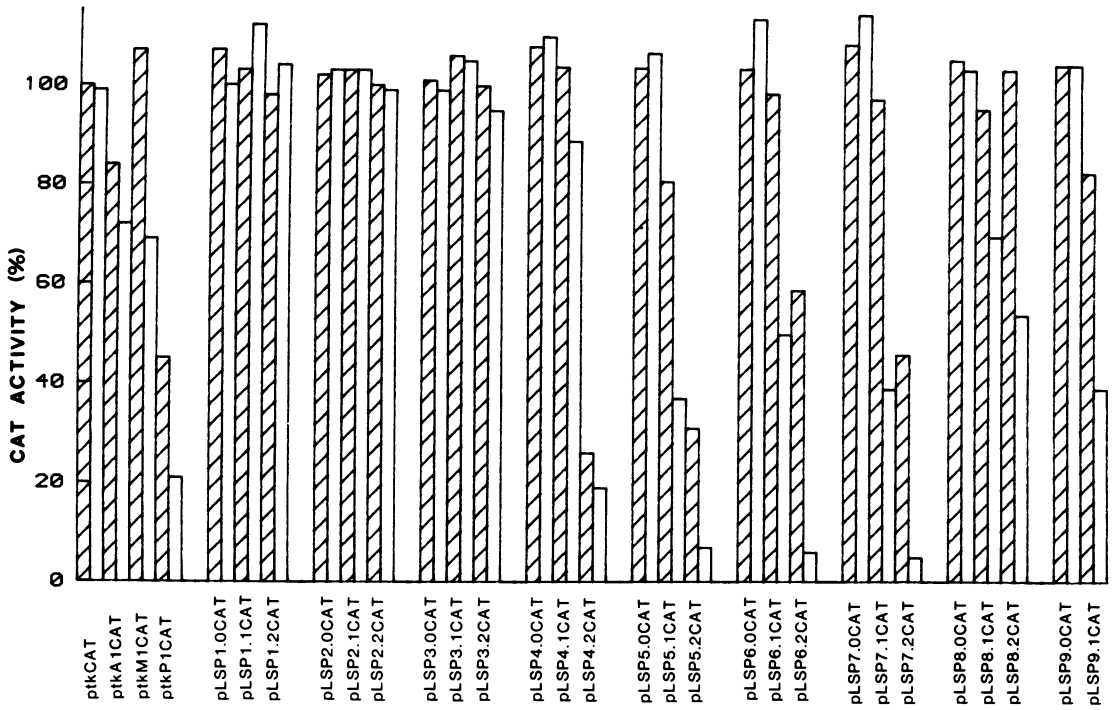

Fig. 6. Repression of CAT activity from LO-promoters in cells expressing LexA protein. Filled bars are CAT activity in L^{tk^-} cells. Open bars are CAT activity in L^{LexA^+} cells. The CAT activity measured after transfection with each plasmid is expressed as a percentage of the activity of the reference plasmid ptkCAT in L^{tk^-} cells.

very carefully determined using spectrophotometric, ethidium bromide fluorescence and Hoechst dye fluorescence techniques to ensure that equal amounts of covalently closed circular plasmid DNA were used for each test. After 24 h the cells were harvested, crude cytosolic cell extracts prepared and assayed for CAT activity. Protein estimation of the cell lysates ensured that equivalent amounts of extract were being used for each assay. The CAT assay method used is described in Materials and methods; all measurements were within the linear response curve of the assay. The levels of CAT activity measured for each plasmid varied by $<5\%$ between duplicate dishes. The results are listed in Table I and displayed graphically in Figure 6.

There was little variation between the activities of the mutant LSP *tk* promoters and that of the wild-type *tk* promoter. Insertion of one or two operator sequences in tandem (a double insert) into the *Bam*HI sites of LSP1 or LSP2, upstream of the essential elements of the promoter, had no effect on promoter activity. Promoters with one operator sequence inserted beween dsII and dsI had activities ranging from 80 to 100% of their reference plasmids. The insertion of two operator sequences in tandem in these positions had a more severe effect in plasmids pLSP4.2CAT and pLSP5.2CAT, reducing promoter activity to 20–30% of their reference plasmids. Single operator inserts between dsI and the TATA box (ps) did not significantly affect promoter activity, but double inserts of the operator sequence in this region reduced activity to between 45 and 70% of the reference plasmid activities. The insertion of one or two operator sequences between the TATA box and the transcription start point had little effect on promoter activity. A single operator sequence inserted downstream of the transcription start point, pLSP9.1CAT and ptkP1CAT, reduced promoter activity to 83 and 45% respectively of the reference plasmids pLSP9.0CAT and ptkCAT. These constructs contain the LexA operator sequence inserted 12 or 18 bp downstream of the transcription start.

To assess repression, the activities of our LO-promoter constructs in L^{tk^-} and L^{LexA^+} cells were directly compared so that the effect of simply inserting operator sequences was eliminated and only repression was measured (Table I). There was no repression of activity from promoters that did not contain LexA operator sequences (the reference plasmids, e.g. ptkCAT, pLSP5.0CAT, etc.) nor from the LO-promoters which contained LexA operator sequences upstream of all the essential region of the promoter (pLSP1.1CAT, pLSP1.2CAT, pLSP2.1CAT and pLSP2.2CAT). Repression of promoters with operator sequences between dsII and dsI (ptkA1CAT, pLSP4.1CAT, pLSP5.1CAT, pLSP4.2CAT and pLSP5.2CAT) increased as the position of the operator more closely approached dsI and the levels of repression were greater when two operators had been inserted in tandem. Promoters with LexA operator sequences inserted between the TATA box (ps) and the Sp1-binding site (dsI) immediately upstream (pLSP6.1CAT, pLSP6.2CAT, pLSP7.1CAT and pLSP7.2CAT) were subject to the greatest repression. In each case repression was greater when two operators had been inserted in tandem: the activity of these promoters was repressed by 90% in L^{LexA^+} cells. The promoters of ptkM1CAT, pLSP8.1CAT and pLSP8.2CAT in which the operator inserts are between the TATA box and the transcription start point were also subject to repression, reducing their activities by ~30% for a single operator insert and by 50% when two operators had been inserted in tandem. The activities of the promoters of pLSP9.1CAT and ptkP1CAT, with single operators inserted downstream of the transcription start point, were also repressed, by 50%.

Discussion

We have used the wild-type *tk* promoter and nine mutant linker-scanning promoters (LSP1–9; McKnight, 1983) to construct LO-promoters containing one, or two LexA operator sequences in tandem, in 12 different positions in the *tk* promoter (Figure 4). The effects of the insertion of the operator sequences on *tk* promoter activity were estimated by comparing the levels of CAT expression from the LO-promoters with those of their reference plasmids, which did not contain LexA operator sequences, in L^{tk^-} cells. Repression by LexA protein was assessed by directly comparing the activities of the LO-promoters in L^{tk^-} and L^{LexA^+} cells.

As expected, the insertion of operator sequences upstream of the essential regions of the promoter, as defined by Coen *et al.* (1986) and McKnight (1983), did not affect the activity of the promoter, and there was no detectable repression from those LO-promoters (pLSP1.1CAT, pLSP1.2CAT, pLSP2.1CAT and pLSP2.2CAT). This result would be predicted if repression by LexA in mammalian cells is due to steric hindrance of either the assembly or progression of the transcription apparatus by bound repressor, as protein bound to operators upstream of the essential regions of the promoter would not interfere with the binding of transacting factors essential for promoter activity.

Insertion of a single operator sequence between dsII and dsI resulted in 80–100% of wild-type promoter activity in L^{tk^-} cells. The insertion of two operator sequences in tandem in this region (pLSP4.2CAT and pLSP5.2CAT) had a more severe effect, reducing promoter activity by 70%. Failure to observe repression from the LSP3 LO-promoters (pLSP3.1CAT and pLSP3.2CAT) was surprising, as one might predict that binding of LexA protein at this point would interfere with the binding of CAAT-binding protein to dsII (Graves *et al.*, 1986). It might be the case that the CAAT transcription factor binds the DNA with a higher affinity than the LexA protein and effectively competes with the binding of LexA protein. As the position of LexA operator insertions more closely approached the Sp1 binding site of dsI we observed increasing levels of repression (ptkA1, pLSP4.1CAT, pLSP4.2CAT, pLSP5.1CAT and pLSP5.2CAT), which was greatest for those LO-promoters containing two operators inserted in tandem. This suggests that we are observing increasing interference by bound LexA protein with the binding of the Sp1 transcription factor.

Insertion of a single operator sequence between dsI and the TATA box (pLSP6.1CAT and pLSP7.1CAT) did not affect promoter activity in L^{tk^-} cells, but insertion of two operator sequences in tandem in this region (pLSP6.2CAT and pLSP7.2CAT) reduced activity by 50%. This could be due to a detrimental increase in the spacing between the essential elements of the promoter or to an alteration of the relative orientation of the essential promoter elements along the DNA double helix. Takahashi *et al.* (1986) reported that the transcriptional activity of the SV40 early promoter is decreased more by the insertion of DNA equal to non-integral turns of the helix than by the insertion of DNA equal to integral turns of the helix between essential elements of the promoter. Similar observations have been made by Cohen and Meselson (1988) for the *Drosophila* heat shock promoter, *hsp70*. One LexA operator sequence in an LO-promoter represents the insertion of 22 bp of additional DNA, equivalent to approximately two turns of the DNA helix (one turn of the DNA helix $\simeq$ 10.5 bp), whereas the insertion of two operator sequences in tandem represents the insertion of 48 bp of DNA, equal to a non-integral number of turns (4.6). Repression of these LO-promoters in L^{LexA^+} cells was the greatest observed out of all the constructs, 50–60% for a single operator insertion (pLSP6.1CAT and pLSP7.1CAT) and 90% with two operators inserted in tandem (pLSP6.2CAT and pLSP7.2CAT). It appears that this region is the most sensitive to repression by LexA. Presumably the binding of the repressor in this region causes the greatest interference with either the assembly and/or the progress of the transcription apparatus.

Promoters containing one or two operators inserted between the TATA box and the transcription start point (ptkM1CAT, pLSP8.1CAT and pLSP8.2CAT) exhibit the same level of CAT expression as the wild-type *tk* promoter. We would anticipate that these insertions would result in a shift in the transcription start point to maintain a distance of 25–30 bp between the TATA box and the point of initiation of transcription (Benoist and Chambon, 1981; McKnight and Kingsbury, 1982; Dierks *et al.*, 1983; Hu and Davidson, 1987). If so, there was no detectable effect on translatability of the resulting mRNA. The activity of these LO-promoter was also repressed in L^{LexA^+} cells, by 30–50%.

The insertion of LexA operator sequences downstream of the transcription start point (ptkP1CAT and pLSP9.1CAT) resulted in a reduction in promoter activity of 20–50%. The 20-bp operator sequence has dyad symmetry which could result in the formation of a hairpin structure in the 5′ untranslated region of the mRNA affecting the efficiency of mRNA utilization. Interestingly we also observed repression of these LO-promoter constructs which contained operator inserts 11 and 16 bp downstream of the transcription start point. This could be due either to the binding of LexA competing with the initiation of transcription or to transcription termination caused by the binding of the repressor protein.

In general, repression was greater when two operators were inserted in tandem than for single copy insertions, a phenomenon also observed by Hu and Davidson (1987). The most efficient repression was observed when the LexA operators were inserted between the Sp1 binding site of dsI and the TATA box of the *tk* promoter. We assume that the repressor protein LexA must dimerize and enter the nucleus passively, either through nuclear pores or during dissolution of the nuclear membrane. In spite of the degree of chromatin structure thought to be associated with transiently expressed plasmid DNA in mammalian cells (Reeves *et al.*, 1985), the repressor must recognize and bind to its operator sequence and presumably sterically interfere with the transcription apparatus to repress expression from the target promoter.

Hu and Davidson (1987) have also demonstrated the use of an *E.coli* repressor, LacI, to down-regulate gene expression in mammalian cells. They used an expression plasmid very similar to pRSVLexA, with the *LacI* gene downstream of the RSVLTR promoter and 3′ SV40 polyadenylation signals. Their target gene, also CAT, was linked to the SV40 promoter with the MSV enhancer upstream. They observed reductions in promoter activity due to the insertion of one or two operator sequences between the essential regions of the SV40 promoter similar to those we

observed for the *tk* promoter. They suggested that reduction in promoter activity was due to increased spacing rather than orientation of the helix as the number of bases inserted was approximately an integral number of helix turns. In the cell line expressing LacI they observed 8- to 12-fold repression with a single operator insert and 24- to 48-fold repression with double inserts in tandem. They were also able to alleviate repression (60–80%) using the inducer IPTG.

Brown *et al.* (1987) have also demonstrated the use of the *E.coli* LacI repressor, in transient expression experiments, to repress gene expression in mammalian cells. This group also used the SV40 promoter for insertion of the LacI operator sequence between the TATA box and the transcription start site, using the synthetic LacI operator sequence which binds LacI repressor eight times more tightly than the wild-type operator. They achieved $\leq$12-fold repression in cotransfection experiments in CV-1P cells, and were able to alleviate repression using IPTG.

The levels of repression we have observed for LexA compare very favourably with those observed for LacI by Hu and Davidson (1987) and Brown *et al.* (1987), taking into account the >1000-fold higher affinity of the LacI repressor for its wild-type operator sequence ($K_d \simeq 10^{-13}$ M; Riggs *et al.*, 1970) compared with the affinity of the LexA repressor for its operator sequence ($K_d \simeq 10^{-9}$ M; Brent and Ptashne, 1984). This could be due to the 28-fold higher levels of expression of LexA repressor protein in our L^{LexA+} cell line (estimated at 7×10^5 molecules/cell) compared with the $LacI^+$ cell line described by Hu and Davidson, in which they estimated the concentration of LacI repressor at 2.5×10^4 molecules/cell. Although neither LacI nor LexA contain any apparent nuclear localization signal, the smaller size of the LexA dimer (mol. wt 46 000) compared with that of the LacI tetramer (mol. wt 150 000) might facilitate its entry into the nucleus.

Materials and methods

Molecular biology

Standard techniques of molecular biology, e.g. plasmid DNA purification, the use of DNA modifying enzymes and gel electrophoresis, were as described by Maniatis *et al.* (1982).

Construction of the LexA expression vector pRSVLexA

The expression vector pRSVLexA is derived from the vector pRSVCAT (Gorman, 1985). This vector contains a *Hin*dIII site immediately 5′ to the CAT gene, downstream of the RSVLTR and an *Nco*I site towards the end of the CAT coding sequence. The DNA between these sites was replaced by a synthetic oligonucleotide linker with sticky ends for the *Hin*dIII and *Nco*I sites. The *Lex*A gene was obtained from Roger Brent as a 950-bp *Hin*dIII fragment (Brent and Ptashne, 1984). This was inserted at the *Hin*dIII site downstream of the RSVLTR to create the LexA expression vector pRSVLexA (Figure 1).

Construction of tkCAT target plasmids ± LexA operator sequences inserted into the tk promoter

Figure 5A illustrates our basic reporter gene plasmid pUCCAT which was constructed by inserting a *Bam*HI–*Xba*I cassette containing the Tn*9* CAT gene fused at the 3′ end to HSV *tk* polyadenylation signals into pUC18. This vector allowed the insertion of the wild-type *tk* promoter or any of its mutant variants, plus or minus LexA operator sequences, upstream of the CAT cassette as *Sst*I–*Bgl*II fragments inserted between the *Sst*I and *Bam*HI sites of pUCCAT. Figure 5B illustrates the basic reference plasmid ptkCAT which contains the wild-type *tk* promoter.

For the insertion of LexA operator sequences the wild-type *tk* promoter was excised from the plasmid pTK1 (Lang *et al.*, 1984) as a 315-bp blunt-ended *Rsa*I–*Pvu*II fragment and inserted into the *Sma*I site of M13 mp18, the *Eco*RI and *Pst*I sites of which had previously been destroyed by filling in and trimming the sticky ends respectively. The LS mutant promoters were excised from their parent plasmid (McKnight, 1983) as *Rsa*I fragments (~373 bp). These were inserted into M13 mp18 which had been cut with *Bam*HI and blunt-ended by filling in the sticky ends.

LexA operator sequences were constructed using synthetic oligonucleotides which when annealed and phosphorylated yielded the necessary cohesive ends. These were cloned into the various M13 *tk* promoter vectors cut with the appropriate restriction enzyme and checked by DNA sequencing to verify the operator sequence, the position of insertion and the number of operators inserted. We have used the synthetic LexA operator sequence described by Brent and Ptashne (1984). Four different operator sequences were synthesized incorporating the four different cohesive ends required for insertion into the unique sites in the wild-type *tk* promoter (*Asu*II, *Mlu*I and *Pst*I) and the *Bam*HI sites of the LS mutant promoters. Different concentrations of operator were ligated with M13 *tk* promoter constructs cut at the appropriate site and dephosphorylated to favour insertion of operator sequences. The ligated DNA was used to transform JM101 and the resulting plaques picked onto Hybond N filters. Colonies which arose from the plaques were screened by hybridization of the operator sequences end labelled with ^{32}P. Positive clones were used to grow up M13 phage and prepare template for DNA sequencing. Single and double inserts of the LexA operator into various positions within the *tk* promoter have been constructed.

We constructed reference plasmids for measuring the wild-type transcriptional activity of the *tk* promoter and the LS mutant promoters with no inserted operator sequences. The promoters were excised from the appropriate M13 constructs as *Sst*I–*Bgl*II fragments (~320 bp) and inserted into pUCCAT cut with *Sst*I and *Bam*HI. These plasmids are called ptkCAT and pLSP1.0–pLSP9.0.

Similarly, the LO-promoters were excised from M13 as *Sst*I–*Bgl*II fragments and ligated into pUCCAT cut with *Sst*I and *Bam*HI. These plasmids were named according to which site contained the operator(s) as indicated in Table I.

Cell culture

L-cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% antibiotic antimycotic solution (Gibco) in 5% CO_2 at 37°C. Cells were removed from dishes for seeding or harvesting using Gibco trypsin–EDTA solution (0.05% trypsin).

Transfection of cells with DNA

We routinely used the calcium phosphate precipitate method of transfection (Gorman, 1985). Cells were seeded at 1×10^6/9-cm dish. After 18–24 h DNA precipitates were added to the dishes and left on the cells for 6 h; the cells were then subjected to 20% glycerol shock and incubated in medium plus sodium butyrate (7 mM) overnight. This was replaced by non-selective medium after 15 h. For transient expression experiments the cells were harvested after 6 h in non-selective medium, 24 h after transfection.

For the isolation of stable cell lines expressing LexA the medium was replaced after 24 h with HAT selection medium (Gorman, 1985). The selection medium was changed every 3–4 days until resistant colonies were visible (after 2–3 weeks). Colonies were picked and grown on to provide enough cells to freeze down five vials of cells as a stock and to prepare cell extracts for Western blots.

For the assay of CAT activity from LO-promoter constructs and their reference plasmids L^{tk-} or L^{LexA+} cells were transfected with DNAs in transient expression experiments. In a standard transfection duplicate 9-cm dishes containing 1×10^6 cells were transfected with 10 μg of test DNA plus 10 μg carrier DNA (salmon sperm) as described above. Five to seven hours after replacement of sodium butyrate medium with non-selective medium, during which time CAT activity was observed to be maximal, the cells were harvested and either stored as PBS washed cell pellets at −70°C or used immediately to make cell extracts for the assay of CAT activity as described below.

Western blot analysis

Preparation of cell extracts. Cell pellets ($1–5 \times 10^6$ cells) were resuspended in 50 μl lysis buffer (0.25 M Tris–Hcl, pH 7.8, 0.1 mM EDTA, 0.3 mM DTT, 1 mM PMSF, 0.5% NP-40), kept on ice for 10 min and then centrifuged 5 min at 12 000 *g* to pellet nuclei and cell debris. The supernatants (crude cytosolic protein preparations) were removed, placed in clean tubes and stored at −70°C or used immediately for PAGE. Proteins from cell extracts (~50 μg) were separated in 12.5% acrylamide gels in the presence of SDS in Tris–glycine buffers (Maniatis *et al.*, 1982). Proteins were transferred from the gel to nitrocellulose filters using electroblotting, with cooling, at 80 V for 3 h in 0.025 M Trizma base, 0.18 M glycine, 25% methanol. Protein transfer was checked by staining the filter with

Ponceau S (BDH). LexA protein was detected using horseradish peroxidase antibody conjugate as described by Jones (1987). Filters were incubated first with anti-LexA antiserum (1:500), then with an excess of swine anti-rabbit immunoglobulin antibody and finally with a soluble complex of horseradish peroxidase and rabbit anti-horseradish peroxidase antibody (secondary antibodies were from Dakopatts). The complex was visualized using a solution of DAB (3,4,3′,4′-tetra-amino biphenyl hydrochloride) and hydrogen peroxide.

CAT assays

Cell extracts were prepared by resuspending cells in 50 μl 0.25 M Tris−HCl, pH 7.8, and disrupting the cells by repeated freezing and thawing. The extracts were then centrifuged at 12 000 *g* for 5 min to remove cell debris and nuclei and the supernatants (40 μl) used as crude cytosolic preparations. Aliquots of these extracts (20 μl) were assayed for CAT activity using a nonchromatographic assay (Sleigh, 1986) in which reaction mixes contained [^{14}C]acetyl coenzyme A and unlabelled chloramphenicol. [^{14}C]Acetylated chloramphenicol produced by CAT enzyme activity was extracted from the reaction mix into 500 μl ethyl acetate which was added to 5 ml scintillation fluid and counted. This assay is more sensitive and more easily quantitated than the widely used TLC assay using [^{14}C]acetylated chloramphenicol (Gorman, 1985). Protein estimations of cell lysates were performed using the method of Lowry *et al.* (1951).

Southern blots

DNA was prepared from 30 9-cm dishes of 50% confluent cells. The cells were lysed in 0.5% NP-40 and the nuclei pelleted by centrifugation. The nuclei were resuspended and treated with proteinase K followed by phenol−chloroform extraction and ethanol precipitation. A DNase-free RNase treatment was included. Southern blotting and preparation of radioactively labelled probes were as described by Maniatis *et al.* (1982).

Northern blots

Total RNA was prepared from 30 9-cm dishes of 50% confluent cells. The cells were lysed in 0.5% NP-40, nuclei and cell debris removed by centrifugation and the supernatants subjected to phenol−chloroform extraction and ethanol precipitation using lithium acetate. Northern blotting and preparation of radioactively labelled probes were as described by Maniatis *et al.* (1982).

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