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The Inducible *lac* Operator–Repressor System Is Functional in Mammalian Cells

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Summary

We have investigated the use of the Escherichia coli *lac* operator–repressor system to regulate expression of transfected genes in mammalian cells. We show that *lac* repressor produced in mouse L cells by transfection of a *lacI* expression vector blocks transcription of an MSV-CAT fusion gene when the *lac* operator is inserted at any one of the following sites within the promoter region: between the initiation codon (ATG) and the transcription start site; between the transcription start and TATA box regions; or upstream of the TATA box region. This last result suggests that the repressor may prevent protein–protein interactions involved in transcription activation. The inducer IPTG causes a marked derepression of CAT expression. The *lac* repressor–operator complex may be useful as an on/off "switch" in the regulation of gene expression for gene transfer experiments.

Introduction

Emerging evidence indicates that one of the major mechanisms for control of gene expression in eukaryotes, just as in prokaryotes, involves binding of a regulatory protein to a specific DNA sequence that it recognizes (for reviews see Dynan and Tjian, 1985; McKnight and Tjian, 1986). Generally speaking, these regulatory mechanisms are somewhat more complex in eukaryotes than in prokaryotes in that they more frequently involve interactions between several different proteins each binding to a distinct short sequence in the regulatory region of the gene (Ptashne, 1986; Davidson et al., 1986).

Repression by binding of the *lac* repressor to the *lac* operator and derepression by action of inducers such as isopropyl β-D-thiogalactoside (IPTG) is one of the most thoroughly studied and best understood examples of a protein–nucleic acid interaction that regulates transcription of a gene in Escherichia coli (Beckwith and Zipser, 1970; Miller and Reznikoff, 1980). The goal of the present research was to determine whether a suitably engineered *lac* repressor–operator system would be functional in animal cells just as in E. coli. If effective, such a system would be a useful addition to the several eukaryotic inducible promoter systems now used for achieving regulated expression of genes introduced into eukaryotic cells by gene transfer. These latter include heat shock inducible promoters (Wu, 1984; Topol et al., 1985), the glucocorticoid-inducible mouse mammary tumor virus long terminal repeat (LTR) (see Ringold, 1983, for review), the metal-ion-inducible metallothionein promoter (Mayo et al., 1982), and poly(IC)-inducible interferon promoters (Goodbourn et al., 1985; Ryals et al., 1985). In addition, information about steric interactions between two eukaryotic regulatory proteins, each binding to its specific sequence in a promoter region, might be provided by observing the effects of *lac* repressor binding to an operator sequence inserted in the vicinity of the two interacting regulating sequences.

The *lac* repressor is a homotetramer containing four polypeptide chains, each of molecular weight 38 kd. To be functional in a eukaryotic cell, this protein would have to self-assemble after synthesis of the monomer units in the cytoplasm, migrate to the nucleus, and bind to an operator sequence that was present in some sort of a chromatin structure characteristic of eukaryotic DNA. To be useful the system would have to be inducible by IPTG added to the external medium. It was not known beforehand whether all this would occur. To our knowledge, the only instance in which a prokaryotic regulatory protein has been shown to bind to its specific recognition sequence in a eukaryotic cell is for the E. coli *lexA* protein to bind to its operator in yeast (Brent and Ptashne, 1984).

Results

Overview

Our overall strategy is depicted in Figure 1. By mutation of the initiator GTG to ATG, the *lacI* coding sequence has been modified so that it is expected to be translated in a eukaryotic cell. It was then inserted into a eukaryotic expression vector driven by the Rous sarcoma virus LTR (RSV LTR) as a promoter. A mouse L cell line denoted LI-1, in which functional repressor is expressed at moderate levels, was selected (Figure 1A).

The *lac* operator sequence has been inserted into selected sites around the promoter element or transcription start site of a standard DNA construction, pSM12, in which the chloramphenicol acetyltransferase (CAT) coding sequence is transcribed under the control of a chimeric promoter consisting of part of the SV40 early promoter coupled to the Moloney sarcoma virus (MSV) enhancer (Laimins et al., 1984). SV40 RNA processing signals are provided at the 3′ end of the gene. This particular construction was chosen because the effect of the MSV enhancer in augmenting expression had already been studied. Furthermore, a series of plasmids with the enhancer in different positions relative to the promoter has been made and studied by Laimins et al. (1984); we anticipate, in future studies, measuring the effect of the position of the *lac* operator relative to the enhancer and the promoter in these several constructions.

As summarized in Figure 1B, we have studied CAT gene expression, both by enzyme assays and RNA blot analysis, after introducing the plasmids by a transient transfection method into the repressor-positive LI-1 cells and, as a control, into the parental L cells. The effect of the inducer IPTG has been measured. Several specific CAT construc-

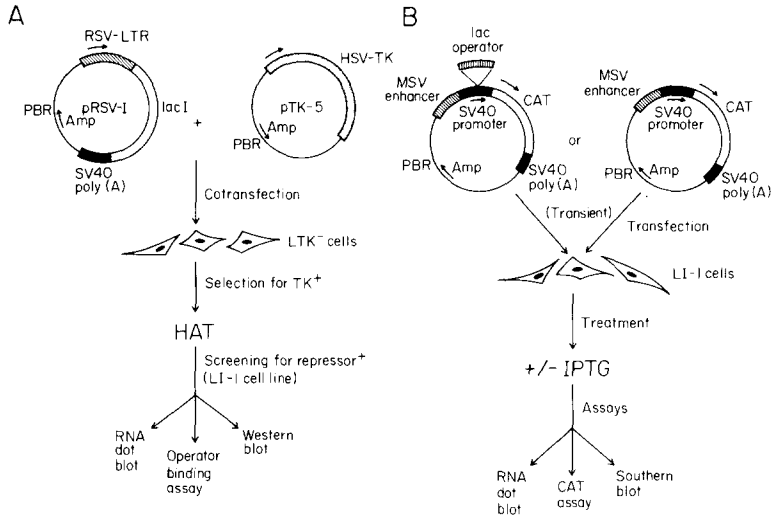


Figure 1. Schematic Representation of the Expression of *lac* Repressor and Its Functional Assay

(A) An expression vector containing an altered *lacI* gene under the control of the RSV LTR was cotransfected into LTK⁻ cells with a plasmid pTK-5, which carries the HSV-1 *tk* gene. The transfected cells were cultured in HAT selective medium, and clonal isolates were screened for the presence of stably expressed *lacI* mRNA and repressor protein (see Experimental Procedures). (B) In the functional assay, an MSV–CAT fusion gene or its derivatives containing *lac* operator sequence was transfected into the repressor producing cells (LI-1). The cells were subsequently cultured in the presence or absence of IPTG. The transient expression of the CAT gene was monitored by the CAT assay and RNA blot analysis, and nuclear DNA uptake was quantitated by Southern blot analysis.

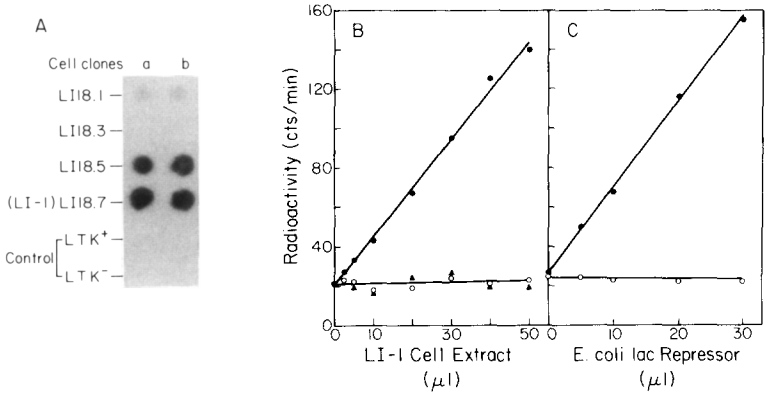


Figure 2. Expression of *lacI* mRNA and *lac* Repressor Protein

(A) RNA dot-blot analyses were performed using total cytoplasmic RNA extracted from L cells (HAT-resistant clones) that had been transfected 2 weeks (a) and 2 months (b) previously with the plasmids pRSV-I and pTK-5. RNA extracted from nontransfected LTK⁻ cells and those transfected with pTK-5 plasmid (TK⁺ cells) were also performed as controls. Equal amounts (4 μg per dot) of each RNA sample were transferred to a filter and were subsequently hybridized with an SP6-generated antisense *lacI* probe. (B) Aliquots of crude cytoplasmic extracts (approximately 2.4 mg/ml) from LI-1 cells and ^{32}P-end-labeled 40 bp operator DNA (2×10^{-12} M) were equilibrated in the presence of 20 μg/ml sonicated calf thymus DNA in standard binding buffer at room temperature (Lin and Riggs, 1972). The reaction was divided into three portions, and each sample was filtered through a nitrocellulose membrane filter (BA85, Schleicher & Schuell); the filters were washed with buffer and counted for radioactivity. No background radioactivity has been subtracted. (●) No IPTG; (○) in the presence of 5 mM IPTG; (▲) cellular extracts from LTK⁻ cells used as a control in the absence of IPTG. (C) Standard binding curve for E. coli *lac* repressor (approximately 27 ng/ml) and operator DNA. The binding assay was performed as described above. No background radioactivity has been subtracted. (●) No IPTG; (○) in the presence of 5 mM IPTG.

tions have been studied. As noted above, the parental plasmid, pSM12, contains a gene consisting of an MSV enhancer, the SV40 promoter, the CAT coding region, the SV40 small t intron, and an SV40 poly(A) addition site. This construction is expected to be transcriptionally active in repressor-positive cells because it does not carry the *lac* operator sequence. The constructions with an operator insert contain: one or several tandem operator insertions between the initiation codon and the transcription start point, designated as pSMAO1, pSMAO2, etc.; one or several tandem operator inserts between the transcription start point and the TATA box region, designated as pSMBO1, pSMBO2, etc.; or one or two inserts between the TATA box region and upstream elements (SV40 21 bp repeats), designated as pSMCO1 and pSMCO2, respectively.

It seemed plausible on general grounds that two or more tandem operator insertions might have a greater effect on gene expression than would a single sequence. This expectation is strengthened by the report that the half-lives of complexes of repressor bound to operator-containing plasmids increase with increasing numbers of tandem operators per plasmid (Sadler et al., 1980).

Functional *lac* Repressor Can Be Expressed in Mouse L Cells

The initiation codon of the E. coli *lacI* gene is GTG (Steege, 1977) and is not expected to function in eukaryotes. (It is of interest to note that changing the GTG initiation codon of a *lacZ* fusion gene into ATG actually results in a higher level of expression in E. coli in vivo and in vitro systems [Looman and van Knippenberg, 1986].) Therefore, in order to achieve a moderately high level of expression of the *lac* repressor in animal cells, we have converted the initiation codon of the *lacI* gene to ATG by site directed mutagenesis, and inserted the coding region into a eukaryotic expression vector driven by the RSV LTR and followed by SV40 splicing and polyadenylation signals, to give the plasmid pRSV-I (see Experimental Procedures).

Mouse LTK$^-$ cells were cotransfected with a plasmid (pTK-5) encoding the herpes simplex virus-I thymidine kinase gene (*tk*) plus a 50-fold excess of pRSV-I by the standard calcium phosphate coprecipitation procedure (Wigler et al., 1979; see Experimental Procedures). Stable transfectants were selected by growth in HAT-medium (see Experimental Procedures; Szybalska and Szybalski, 1962). Clonal isolates were screened for the presence of stably expressed *lacI* mRNA and repressor protein. Many clones expressed the repressor gene by these criteria. RNA dot-blot data for several clones and appropriate negative controls are shown in Figure 2A. Further studies have been confined to the cell line LI-1, which is the highest level expresser of the clones isolated.

The tetramer repressor protein produced in E. coli is capable of binding to the operator sequence in vitro. To determine whether repressor that is functional by this criterion is produced in the transfected animal cells, we performed a filter-binding assay for repressor–operator DNA complexes (Lin and Riggs, 1972). Crude extracts prepared from the cotransformed clones were incubated with the purified ^{32}P-end-labeled operator fragment (40 bp), in the presence or absence of IPTG, prior to filtering through a nitrocellulose filter. In addition, purified *lac* repressor protein was used to standardize the operator-binding curve. Figure 2B shows a filter-binding assay of cell extracts from the *lacI*$^+$ LI-1 cells, and demonstrates that these cells synthesize functional repressor protein that specifically binds the operator fragment in the presence of a vast excess of calf thymus DNA just as does the purified E. coli protein. By comparison of the linear binding curves of the extracts and of the purified protein, we estimate that there are 2.5 ($\pm$0.5) $\times$ 10^4 functional repressor molecules per cell. The molecular weight of the tetramer is 150 kd (Gilbert and Müller-Hill, 1970); therefore, assuming 10^{-9} gram of protein per cell, the functional repressor constitutes ca. 6 $\times$ 10^{-6} by weight of total cell protein.

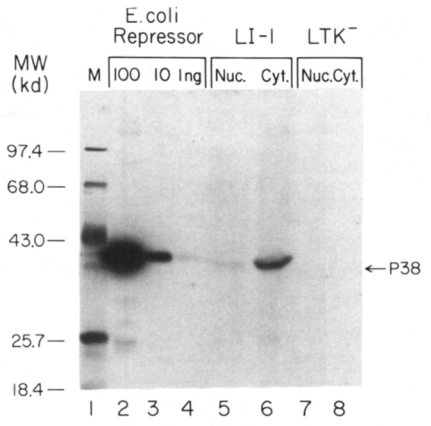


Figure 3. Quantitative Western Blot Analysis of *lac* Repressor in LI-1 Cells

Crude cytoplasmic and nuclear extracts from LI-1 and LTK$^-$ cells (2 $\times$ 10^6) were electrophoresed through an SDS–polyacrylamide gel and were transferred to nitrocellulose. The blot was probed with anti-repressor monoclonal antibody B-2 (Sams et al., 1985) followed by ^{125}I-labeled sheep anti-mouse immunoglobulin. After autoradiography, the bands were quantitated by densitometric scanning. The migration of *lac* repressor monomer (P38) is indicated. The positions of ^{14}C-labeled molecular weight standards are shown in the left margin. Cyt., cytoplasmic fraction; Nuc., nuclear fraction; M, marker proteins.

The *lac* Repressor Is Distributed Between Cytoplasm and Nucleus

Although the *lac* repressor is a well-characterized DNA-binding and regulatory protein in E. coli, it was not known whether it could penetrate the nuclear envelope and how it would distribute between cytoplasm and nucleus in animal cells. To address this question, Western blot analyses were performed on extracts of separated cytoplasmic and nuclear fractions of LI-1 cells, using a repressor-specific monoclonal antibody B-2 (Sams et al., 1985; see Experimental Procedures).

The immunoblots in Figure 3 show that a repressor polypeptide of approximately 38 kd comigrating on an SDS gel with the monomer derived from purified E. coli repressor is present in LI-1 cells but not in LTK$^-$ cells. About 10% of the total repressor was found in the nuclear fraction and 90% in the cytoplasm. The estimates from these gels of total repressor polypeptide present agree well with the amount found by operator binding; thus, most of the repressor polypeptide in LI-1 cells is present as functional tetramer. We therefore estimate that about 2500 repressor molecules are present in the nucleus, where they might be able to act as negative regulatory molecules.

The measured ratio of nuclear to cytoplasmic repressor could be quantitatively distorted by nuclear leakage or disruption and/or by adherence of cytoplasmic material to the external nuclear surface. The gene expression experiments reported below, as well as experiments with stable transformants and experiments by Xenopus oocyte injection methods (unpublished results), all show that there is some biologically active repressor in the nucleus.

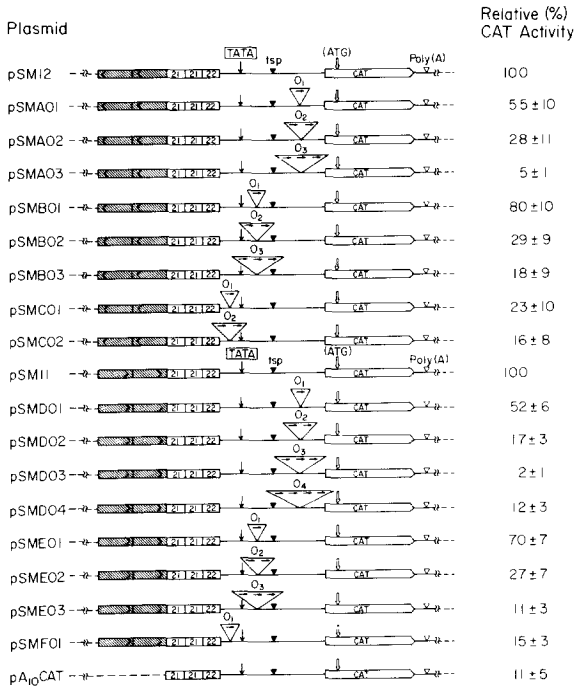


Figure 4. Structural and Functional Properties of Operator Insertions in the Promoter Regions

The organization of the SV40 early promoter region in parental plasmids (pSM12 and pSM11) and in their derivatives containing operator insertions is depicted. The hatched boxes to the left of the SV40 promoter illustrate the MSV enhancer (the 73/72 bp tandem repeats; Laimins et al., 1984). The SV40 21 bp repeats, TATA box, transcription start point (tsp), initiation codon (ATG), CAT-coding sequences (boxed region), and poly(A) addition site are indicated. The positions of single and multiple tandem operators (denoted as horizontal arrows) are shown as triangular inserts, and the copy number of the operator insert is indicated at the top of each triangle: O_1 (one operator), O_2 (two tandem operators), O_3 (three tandem operators), O_4 (four tandem operators). Individual cultures of LTK$^-$ cells were transfected with 4 μg (at 2 μg/ml) of the indicated plasmid DNA by using the DEAE-dextran method, and cell extracts were prepared for assaying CAT activity at 48 hr after transfection. CAT assays were performed at least three times, using equal amount of protein from the cell extracts, under linear assay conditions. The relative CAT activity is quantified as percentage of parental plasmid pSM12 CAT activity. The standard deviations shown on the right were calculated using data from four independent transfection experiments. Transfections and assays were repeated by using two different preparations of the plasmids that showed less than 50% CAT activity in transient expression in LTK$^-$ cells. The control CAT expression was also performed using $pA_{10}CAT$, the plasmid that contains the SV40 promoter elements but no enhancer sequences. The finite but low level of expression observed from this control plasmid has been observed by others (N. Fregien, personal communication). In our hands, the expression ratio for $pA_{10}CAT$ relative to pSM12 varies with amount of DNA and with host cell type. We suspect that transcription from $pA_{10}CAT$ may be due to a cryptic promoter within the vector, as we observe for pUCOCAT (constructed by N. Fregien), which has no eukaryotic promoter (see also Lopata et al., 1986; and Fregien and Davidson, 1986).

Effects of Operator Insertions on Gene Expression in the Absence of Repressor

Before studying the possible effects of repressor binding to operator sequences in the several plasmid constructions in vivo, it was necessary to determine the extent to which operator inserts affect gene expression in the absence of repressor. Accordingly, transient expression studies were carried out with these plasmids in LTK$^-$ cells. Figure 4 shows that insertion of a single operator between the initiation codon and the transcription start (pSMAO1 and pSMDO1) caused a 45%–48% decrease in CAT activity with respect to the parental plasmid (pSM12 and pSM11, respectively); two tandem operators (pSMAO2 and pSMDO2) caused a 72%–83% decrease in CAT activity; and three tandem operators (pSMAO3 and pSMDO3) almost eliminated CAT activity. Similarly, insertion of a single operator between the transcription start and the TATA box (pSMBO1 and pSMEO1) decreased CAT activity approximately 20%–30%; two tandem operators (pSMBO2 and pSMEO2) decreased CAT activity about 70%; and three tandem operators (pSMBO3 and pSMEO3) decreased CAT activity about 80%–90%. In contrast, insertion of a single operator between the TATA box and the upstream SV40 21 bp repeats (pSMEO1 and pSMFO1) resulted in a 77%–85% reduction in CAT activity. These latter results are in agreement with a previous study showing that insertion of DNA segments of increasing length between 21 bp repeats and the TATA box region leads to a significant decrease in the amount of RNA transcript initiated at the major early-early start sites (Takahashi et al., 1986). (It may be noted, in Figure 4, that we have studied operator insertions into constructions pSM11 and pSM12 of Laimins et al. (1984) with both orientations of the enhancer relative to the transcription direction. Our later studies in LI-1 cells have been restricted to a subset of these plasmids.)

The *lac* Repressor-Operator Complex Functions As a Regulatable Switch in Animal Cells

To examine the functional capability of the expressed repressor as a negative transcription regulator in mammalian cells, we performed CAT assays with extracts of LI-1 cells obtained after transfection with the reporter genes (see Experimental Procedures). As shown in Figure 5A, derivatives of the parental plasmid pSM12 containing a single operator between the initiation codon and the transcription start (pSMAO1, lane 2), between the transcription start and the TATA box regions (pSMBO1, lane 4), and between the TATA box and the SV40 21 bp repeats (pSMCO1, lane 6) were repressed 8–12-fold in the repressor-positive LI-1 cells relative to the same plasmids in the repressor-negative L cells. All pSM12 promoter derivatives containing two tandem operators (Figure 5A, pSMAO2 and pSMBO2) were repressed more efficiently (about 30-fold) than corresponding derivatives that contained only a single operator. In summary, in the presence of repressor, CAT enzymatic activity was reduced by 87%–92% for the single-operator-inserted genes and by 96%–98% for the two-operator-inserted genes.

To determine whether CAT gene expression was regulated at the level of transcription, we isolated total cytoplasmic RNA from transiently transfected LI-1 cells and determined the concentrations of CAT RNA by RNA dot-blot hybridization (Figure 6B). The relative amounts of CAT-specific RNA paralleled the levels of CAT enzyme activity in the transfected cells.

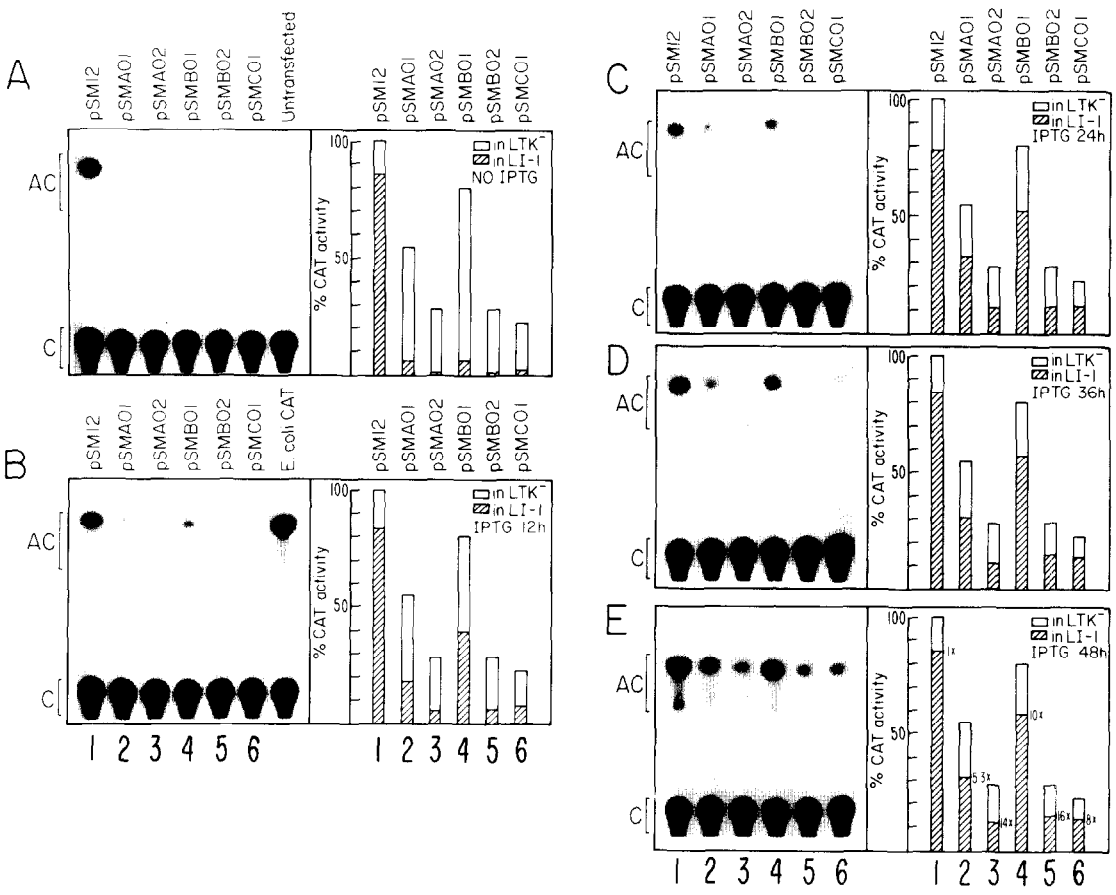


Figure 5. Repression and Induction of CAT Activity in Repressor-Producing Cells (LI-1)

Subconfluent LI-1 cells were transfected with 4 μg (at 2 μg/ml) of plasmid DNA, as indicated in each lane, by using the DEAE-dextran protocol. After transfection, cells were cultured for 48 hr in galatose-substituted DME media (see Results and Experimental Procedures) under the following conditions: (A) no IPTG was included; (B) IPTG was added to a 20 mM final concentration at 36 hr after transfection, and cells were continuously cultured for 12 hr; (C) IPTG added at 24 hr after transfection, and cells were cultured for another 24 hrs; (D) IPTG added at 12 hr after transfection, and cells were cultured for additional 36 hr; and (E) IPTG added right after transfection, and cells were cultured for 48 hr. Analyses of CAT activity were carried out 48 hr following transfection as previously described. The relative CAT activity is normalized as a percentage of the parental plasmid's (pSM12) CAT activity in LTK⁻ cells. Shown to the right of these CAT assays (A–E), the level of CAT expression from the indicated plasmid constructs in LI-1 cells (hatched bars) was compared with that in LTK⁻ (empty bars) which is shown in Figure 4. The magnitude of induction in 48 hr as shown in (E) was individually quantified by dividing the % CAT activity of each in (E) by that observed in (A) in LI-1 cells. As an additional negative control, when the operator was inserted downstream of the poly(A) site within the vector, CAT expression was not repressed in LI-1 cells.

These experiments are most plausibly interpreted as showing that binding of the repressor to an operator sequence inserted upstream of the transcription start point at the several sites that we have used inhibits either binding of RNA polymerase II or successful initiation of transcription. Inhibition by repressor bound to the operator at a site that begins 42 nucleotides downstream of the start point may be due to steric interference with initiation or, more plausibly, may interfere with propagation of the transcription complex. The latter view is consistent with the evidence that the *lac* repressor–operator complex is an efficient terminator of transcription both in vivo and in vitro in a prokaryotic expression system (Deuschle et al., 1986).

In E. coli the β-galactoside IPTG is an efficient inducer of *lac* gene expression from the repressed state. Since we have shown that repressor protein can act on operator sequences in animal cells, we next asked whether IPTG in the external medium can relieve this repression. We therefore measured CAT enzyme activity as a function of time after the addition of IPTG to the medium. Transfected LI-1 cells were grown in the galactose-substituted DME medium (see below) in the presence of 20 mM IPTG for 12 to 48 hr. At various times thereafter, cytoplasmic extracts were prepared, and CAT enzyme activity was measured. As shown in Figures 5B–5E, treatment of the transfected cells with IPTG induces expression of CAT activity. Induction occurred rather slowly, being half-maximal after 12 hr (Figure 5B) and nearing plateau by 24 hr (Figure 5C). The level of induction is ca. 5-fold for the construct pSMAO1 (Figure 5E, lane 2), 14-fold for pSMAO2 (lane 3), 10-fold for pSMBO1 (lane 4), 16-fold for pSMBO2 (lane 5), and 8-fold for pSMCO1 (lane 6). However, induction levels never returned to the fully derepressed level estimated from expression in LTK⁻ cells. These experiments were carried out with galactose rather than glucose in the tissue culture medium, because galactose is a weak inducer of *lac* repressor whereas glucose is a weak anti-inducer in bacteria and in vitro (Barkley and Bourgeois, 1980; Riggs et al., 1970). The magnitude of induction in the presence of a given concentration of IPTG in the galactose-substituted

DME media was about 2-fold higher than that in the glucose-containing DME media. There was no detectable induction in the galactose-substituted DME media in the absence of IPTG. In addition, we note that IPTG had no effect on the growth of LI-1 or LTK$^-$ cells.

The effects of IPTG were also monitored by measuring CAT mRNA levels. The RNA dot-blot hybridizations (Figure 6C) show that the amounts of CAT-specific RNA induced corresponded well with the levels of the CAT enzyme induced in transfected cells. The induction ratios in cells transfected with plasmids containing two tandem operators (pSMAO2 and pSMBO2) are greater than those for cells transfected with plasmids containing a single operator (pSMAO1 and pSMBO1). As previously noted, the repressed levels of expression are always lower for the plasmids containing two inserted operators than for the ones with a single operator. In neither case, however, does the maximally induced state reach the level observed for the same plasmids in LTK$^-$ cells, where there is no repressor.

In summary, these results show that the several MSV–CAT fusion genes carrying *lac* operators at different positions within the promoter regions were repressed by the *lac* repressor expressed in LI-1 cells in the absence of IPTG and were derepressed by induction with IPTG.

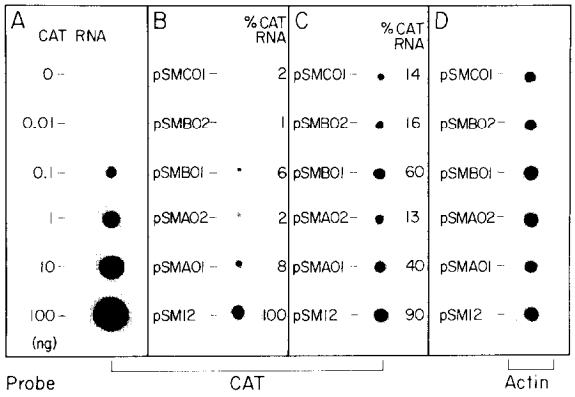


Figure 6. Analysis of CAT RNA Synthesized in the Absence or Presence of IPTG in LI-1 Cells

RNA dot-blot analyses were performed using RNA extracted from LI-1 cells that had been transfected 48 hr previously with the indicated plasmid constructs. RNA samples (4 μg per dot) were transferred to filters and were subsequently hybridized with an SP6-generated antisense CAT probe that hybridizes only to CAT-coding sequences or with an SP6-generated antisense actin probe (Hu et al., 1986). (A) Nontransfected LTK$^-$ cell RNA (2 μg) plus 0, 0.01, 0.1, 1, 10, or 100 ng of CAT RNA that was made by transcription in vitro of an SP6 clone (p64-CAT, which gives sense-strand RNA). (B) RNA from transfected LI-1 cells cultured in the absence of IPTG. (C) From a different transfection, RNA from LI-1 cells cultured in the presence of IPTG as described for Figure 5E. (D) A control experiment of (B) that was probed with an SP6-generated antisense actin probe. Controls show that the amount of total cellular RNA used per dot contributes negligibly to the signal in this assay. The relative CAT RNA in the respective transfectants was calculated after scintillation counting of each sample and comparing the radioactivities to that of the parental plasmid's CAT RNA.

Operator Inserts Immediately Downstream of the TATA Box Shift the Transcription Start Site

The current view on the function of the TATA element in higher eukaryotes is that it acts as a selector for transcriptional initiation sites by defining a fixed number of base pairs downstream at which RNA polymerase begins transcription. Consistent with this view, deletions of the TATA element result in heterogeneous initiation, and deletions that remove sequences downstream from a TATA element result in mRNA initiated from a new site (Grosschedl and Birnsteil, 1980; Benoist and Chambon, 1981; Mathis and Chambon, 1981; Dierks et al., 1981; Ghosh et al., 1981; Kamen et al., 1982; McKnight and Kingsbury, 1982; Dierks et al., 1983). (Note, however, that it has been recently reported that yeast mRNA initiation sites are determined primarily by specific sequences, not by the distance from the TATA element [Chen and Struhl, 1985; Hahn et al., 1985].)

It was therefore important to determine whether the operator insertions in the plasmids studied here would influence the choice of the transcription start site. We therefore performed CAT-specific primer extension experiments with cytoplasmic RNA extracted from LTK$^-$ cells that had been stably transfected with each MSV–CAT fusion gene tested. (Stable transfectants were used because of the greater specific activity of the CAT RNA in these samples.) We used as primer a 36 nucleotide 5′-end-labeled oligonucleotide complementary to a sequence within the CAT gene (see Experimental Procedures). With RNA from cells transfected with the parental plasmid pSM12, the primer extension product was extended to a length of 144 nucleotides, as expected (Figure 7A, lane 3; the relevant sequences are shown in the legend). In the plasmid pSMAO1, an operator segment of length 40 bp had been substituted for a 17 bp StuI–HindIII fragment between the initiation codon and the transcription start site. With the resulting RNA, the DNA primer was extended to about 168 nucleotides (Figure 7A, lane 4). Thus, the distance between the TATA region and the start site of RNA synthesis in this plasmid was the same as that in the parental plasmid pSM12. Furthermore, Figure 7B shows that mRNA from the plasmid pSMCO1 (lane 2), carrying one operator between the TATA region and the SV40 21 bp repeats, has the same 5′ end as the mRNA from the parental plasmid pSM12. Therefore operator insertions downstream of the transcription start site or upstream of the TATA box do not affect the position of the transcription start site. However, when RNA from cells transfected with pSMBO1, containing one operator insertion between the transcription start site and the TATA box, was used as template, the same primer was extended to 184 nucleotides (Figure 7A, lane 2, and see legend). This corresponds to a new start site at a T residue within the operator sequence. This new start site is 25 bp downstream of the residue T_{15} of the TATA box (numbering as shown in Takahashi et al., 1986). Thus, these results support the view that the TATA region has a "fixing" function for the initiation of transcription in mammalian cells.

Transfected Supercoiled DNA Becomes Nicked in LTK$^-$ Cells but Remains Mostly Intact in LI-1 Cells

We sought to confirm that the apparent repressor-mediated repression of operator-containing MSV–CAT

promoters was not caused by variation in plasmid copy number in the transiently transfected cells. Accordingly, total DNA was isolated from nuclei from transfected LI-1 cells by the Hirt procedure (Hirt, 1967) and was analyzed by electrophoresis on 1% agarose gels and subsequent blot hybridization to a ^{32}P-labeled CAT probe. Results presented in Figure 8 show that the yields of the various CAT plasmid DNAs, as determined by Southern blotting, could not account for the differences in RNA expression or CAT enzyme levels. It was quite surprising that most of the transfected supercoiled DNA remained intact after 48 hr in the repressor-producing cells LI-1. Previous studies have shown that in transfection experiments, extracellular DNA that is not washed out when the culture medium is changed is completely degraded over a 22 hr period following transfection (Alwine, 1985). Therefore the DNA studied here, as isolated by the Hirt (1967) procedure, had been incorporated into the cells. In contrast, in other studies it has been reported that most of the transfected supercoiled DNA becomes nicked or linearized during the course of transfection (Weintraub et al., 1986). To investigate this further, we analyzed DNA from transfected LTK$^-$ cells by Southern blotting as described above. Figure 9 shows that after 48 hr the input supercoiled DNA was largely nicked (27%–62%), but only a small fraction (8%–13%) of DNA became linearized. (Nicked molecules were not distinguished from closed-circular relaxed molecules since they comigrated under the electrophoresis conditions used.) Moreover, some transfected DNA (10%–50%) became concatenated. We note that we did not see concatenation of transfected DNA in LI-1 cells (Figure 8), probably because most of the DNA did not become linearized. Thus there is less nicking and cutting of the plasmid DNA in the repressor-positive LI-1 cells than in the repressor-negative LTK$^-$ cells. This phenomenon does not depend on the presence of an operator sequence in the plasmid; it was observed for pSM12 also. A plausible but speculative interpretation of these results is that the plasmids picked up a protective coat of *lac* repressor protein—because of its nonspecific DNA-binding properties (Lin and Riggs, 1972)—during their passage through the cytoplasm to the nucleus, where they became packaged into chromatin as minichromosomes (Cereghini and Yaniv, 1984; Reeves et al., 1985; Weintraub et al., 1986). Furthermore, transfected DNA was found to be mostly unintegrated in LTK$^-$ and in LI-1 cells in accordance with a previous report (Weintraub et al., 1986). The results presented in Figure 9 also indicate that the quantity of transfected DNA taken up and persisting in nuclei was approximately the same for each plasmid,

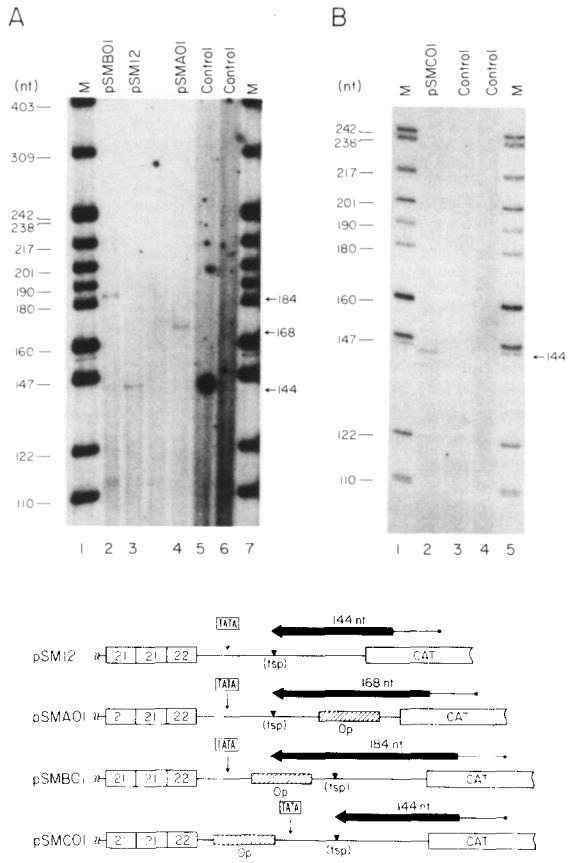
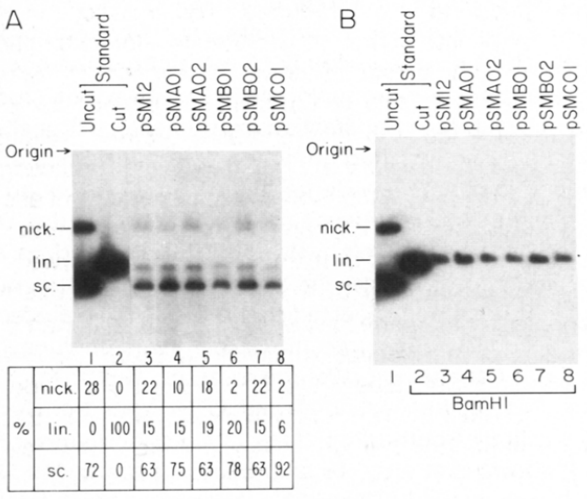


Figure 7. Primer Extension Analysis of Cytoplasmic RNAs Produced by the MSV–CAT Fusion Genes Containing Operator Insertions

(A) The 5′-^{32}P-labeled 36 nucleotide synthetic oligonucleotide complementary to the CAT coding sequence (see Experimental Procedures) was hybridized to approximately 70 μg total cytoplasmic RNA from LTK$^-$ cells transfected with the indicated plasmid constructs. The primer was then extended with unlabeled dNTPs with reverse transcriptase, and the products were fractionated on a 6% polyacrylamide sequencing gel. As control experiments, the same primer was hybridized to either the same amount of total cytoplasmic RNA from nontransfected LTK$^-$ cells (lane 5) or no RNA (lane 6). (B) The same primer was hybridized to approximately 70 μg total cytoplasmic RNA from LTK$^-$ cells transfected with pSMCO1 (lane 2) and then extended as described above. The extended product is displayed on a 6% polyacrylamide sequencing gel. The control experiments were performed as described above (lane 3, RNA from nontransfected LTK$^-$ cells; lane 4, no RNA). ^{32}P-labeled HpaII fragments of pBR322 served as nucleotide size markers (M), which are marked in the left margins. The band due to the extended product is identified by an arrow, and its length is indicated in the right margin of each panel.

Synthesis of the observed cDNA products is depicted schematically below the figure. Symbols are the same as described in the legend to Figure 4. The 40 bp of the *lac* operator insertions is indicated by the hatched box. The primer is shown as a thin line, and extension products as thick arrows. The relevant sequences are shown below:

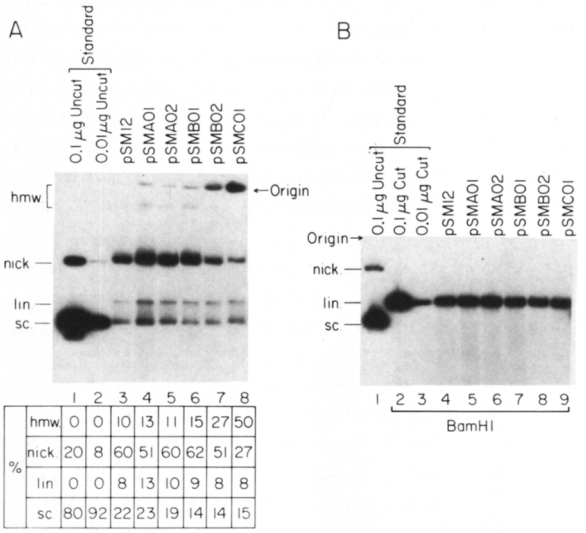
The dashed lines underneath the sequences indicate the extended products, and asterisks denote the sequences deleted in the course of insertion of the *lac* operator sequences.



%		1	2	3	4	5	6	7	8
	nick.	28	0	22	10	18	2	22	2
	lin.	0	100	15	15	19	20	15	6
	sc.	72	0	63	75	63	78	63	92

Figure 8. Southern Blot Analysis of Transfected DNA in the Nuclear Fraction of LI-1 Cells

Total DNA was isolated from nuclei from LI-1 cells transfected 48 hr previously with the indicated plasmids in the absence of IPTG; DNA was analyzed by electrophoresis on 1% agarose gels and subsequent blot hybridization to an SP6-generated CAT probe (from p64-CAT, which gives sense-strand RNA). (A) DNA was undigested; (B) DNA was digested with BamHI prior to electrophoresis. The lanes marked uncut (lane 1) and cut (lane 2) are samples of standard plasmid (0.1 μg of pSM12), either intact or after digestion with BamHI, respectively. Approximately equal amounts of DNA were loaded in each lane. The form of the input DNA is indicated (nick., nicked; lin., linearized; sc., supercoiled), and the relative amount of DNA, as shown below (A), was quantitated by densitometric scanning and then normalized as a percentage of the total CAT DNA.



%		1	2	3	4	5	6	7	8
	hmw.	0	0	10	13	11	15	27	50
	nick	20	8	60	51	60	62	51	27
	lin	0	0	8	13	10	9	8	8
	sc	80	92	22	23	19	14	14	15

Figure 9. Topology of Transfected DNA in Isolated Nuclei of LTK⁻ Cells

Total DNA was isolated from nuclei from transfected LTK⁻ cells at 48 hr after transfection and then analyzed and quantitated as described in Figure 8. (A) Uncut DNA; (B) DNA digested with BamHI before being separated on 1% agarose gels. High molecular weight DNA is indicated as hmw. All other symbols are the same as in Figure 8.

and thus could not account for the differences in CAT expression between the operator-containing derivatives and the parental plasmids.

Discussion

Our overall result is that the bacterial *lac* repressor protein encoded in a suitable expression vector can be synthesized in animal cells, assemble into a tetramer, enter the nucleus, and repress expression of another gene that has one or several *lac* operator sequences inserted into any one of several sites in the promoter region of this plasmid. Derepression can be achieved by exposure of the cells to IPTG.

In eukaryotes, most regulatory proteins are transported from their cytoplasmic site of synthesis and are localized primarily in the nucleus. This relatively strict nuclear accumulation is attributed to the presence in the protein of a distinctive stretch of amino acids that acts as a nuclear-localization signal (Goldfarb et al., 1986; Richardson et al., 1986; and also see Dingwall, 1985, for review).

We observe that about 10% of the total *lac* repressor in LI-1 cells, or about 2500 molecules, is located in the nucleus. Since the nuclear volume is about 6% of the total cell volume in a mammalian cell (Alberts et al., 1983), the average concentration of repressor is about the same in the nucleus and cytoplasm. While this result can be rationalized in a number of ways, we find it rather surprising in view of the relatively strong nonspecific DNA-binding properties of repressor. In any case, with a diploid genome size of 6×10^9 bp, LI-1 cells contain approximately one repressor per 2×10^6 bp of DNA. In E. coli, with a genome size of 4.2×10^6 bp and perhaps two chromosomes per cell, and with ca. 10 repressor molecules per cell (Müller-Hill et al., 1968), there is ca. one repressor per 4.2×10^5 bp. These numbers are not greatly different. It is of interest to note that Chao et al. (1980) have reported that, in vitro, assembly of *lac* operator–containing DNA into nucleosomes with octameric histone cores does not affect specific sequence recognition by repressor, or IPTG-induced dissociation, but that nonspecific binding of repressor to DNA is considerably diminished. Specific recognition presumably shows that the repressor-binding surface of the operator DNA faces outward on the nucleosome. Our results showing specific gene regulation by *lac* repressor but limited overall nuclear localization are totally consistent with their results. We note further that Silver et al. (1986) have reported very recently that the DNA-binding protein *lexA* and a *lexA* chimeric DNA regulatory protein are localized predominantly in the cytoplasm in yeast cells.

The data in Figure 8 show that 48 hr after transfection, there are about 150 6 kb plasmid molecules per nucleus, corresponding to an additional 9×10^5 bp. These DNA molecules have entered the nucleus by passage through the cytoplasm. They may have thus acquired an additional coating of repressor, as suggested by the apparent protection against nicking observed in the LI-1 cells. (However, our preliminary results with cells in which some of the plasmids derived here have been stably transfected into the chromosomal DNA of LI-1 cells are that the levels of repression are about the same. Thus, the phenomena

of repression are not dependent on recent exposure of the DNA to the cytoplasm.)

By comparison of the expression levels of the plasmids in repressor-positive LI-1 cells and repressor negative LTK$^-$ cells, we deduce repression levels of 8–12-fold for a single operator insert and 24–48-fold for two in tandem. This is substantially less than the ratio of induced to repressed expression of the *lac* operon in E. coli, which may be as high as 1000-fold (Gilbert and Müller-Hill, 1970). The reasons for this are unknown. They may be related to the different molecular environments of the operator DNA sequence in eukaryotic chromatin and in a bacterial cell. However, our results are concordant with recent experiments that show approximately 10-fold repression, by the mechanism of transcription termination, where the *lac* operator sequence is inserted downstream of coliphage T5 promoter and assayed in E. coli (Deuschle et al., 1986). Whether the extent of repression could be augmented by increasing the overall level of expression of repressor protein or by adding a eukaryotic nuclear localization signal at an appropriate point in the protein is not known at present.

Furthermore, we note that while a significant level of derepression (60%–80%) can be achieved with IPTG, it is not fully reversible, and it occurs slowly (ca. 24 hr). This limited induction may be related to the limited permeability of the eukaryotic plasma membrane to IPTG, but we have no data bearing on this question. In E. coli, IPTG normally enters through the inducible *lac* permease system (Kennedy, 1970); nevertheless, permease-negative mutants are still inducible for *lacZ* by IPTG, although the inducer uptake rate is reduced (Herzenberg, 1959).

From a practical point of view of an inducible genetic switch, the induction ratios achieved at present with the *lac* operator system are not better than those that have been achieved with heat shock, mouse mammary tumor virus, and metallothionein promoters. There may, however, be some situations and some promoters for which the use of the *lac* operator system is advantageous. Further work may reveal methods for achieving higher induction ratios. It will be of interest to test whether the induction ratios observed here can be increased by applying glucocorticoids, since it has been recently reported that the expression of genes fused downstream of the MSV LTR is stimulated 3- to 5-fold by hormone treatment (DeFranco and Yamamoto, 1986).

Our results on the effects of operator insertions and repressor binding on transcript levels have interesting implications for general mechanisms of regulation of gene expression. Specific cases are discussed below. We observe that inserting either one or two copies of the 40 bp operator sequence between the TATA box and the 21 bp repeats reduces expression by a factor of approximately 5 (pSMCO1 and pSMCO2, Figure 4). Specific transcription factors related to the SP1 factor are believed to bind to the 21 bp repeats and contact a protein bound to the TATA box (for review, see Dynan and Tjian, 1985); the latter, in turn, interacts with RNA polymerase II to activate the transcriptional machinery. When this SP1 factor and the TATA box factor are separated by one or two *lac* operators, transcription rates are reduced. The interactions must now require interaction at a distance—for example, by looping (Ptashne, 1986). Binding of repressor to the operator sequence in this position greatly inhibits transcription; presumably the *lac* repressor protein presents a steric block to the normal protein–protein interactions.

Takahashi et al. (1986) have tested the effects of altering the stereospecific relationships of SV40 promoter elements by constructing insertions of 4 or 15 bp between the 21 bp repeats and the TATA box region. These insertions decrease transcription in vivo more drastically than insertions of 10 or 21 bp because they place the interacting proteins on opposite sides of the DNA helix. The *lac* operator sequence used by us is 40 bp in length, which is approximately an integral number of turns. Thus the effects observed here in the absence of repressor are mainly due to increased distance, not opposite orientation relative to the helix axis.

In agreement with earlier studies, we observed that placement of a 40 bp operator segment between the TATA box and the normal transcription start point changes the start point to a new site, which is again about 25 nucleotides from the TATA box (see legend to Figure 7). In the presence of the repressor, transcription is reduced by a factor of about 12. We were unable to map the start point in the repressed state because of the amount of RNA produced; if it is the same, we assume that the binding of a protein to the TATA box or of RNA polymerase II close to the transcription start point can displace the binding of repressor to the operator.

Finally, binding of repressor to an operator inserted downstream of the transcription start point also inhibits transcription. This is most plausibly interpreted as a block to passage of the transcription complex, in agreement with recent results for E. coli RNA polymerase in vivo and in vitro (Deuschle et al., 1986). In addition, we note that insertion of operator sequences between the transcription start point and the initiation codon may result in the formation of a hairpin structure in the 5′-untranslated region of the mRNA, thus affecting translation initiation. The *lac* operator sequence has extensive bilateral symmetry (Barkley and Bourgeois, 1980). This hypothesis is supported by recent experiments in which hairpin structures were generated upstream of the initiator codon by oligonucleotide insertion, thus leading to a drastic reduction in translation (Kozak, 1986).

Experimental Procedures

Site-Directed Mutagenesis of the *lacI* Gene

The *lacI*^{q} gene contained in plasmid pIQ (J. L. Betz, unpublished results) was excised by EcoRI and HincII digestions, and subcloned into the EcoRI and HincII sites of the vector M13mp19 (Yanisch-Perron et al., 1985). The oligonucleotide 5′-TGGTGAATATGAAACCAG-3′ was employed as a mismatched primer to introduce a G-to-A mutation of the GTG initiator codon. The 5′-phosphorylated mutagenic primer and the M13 primer 5′-ATGCCCCCTGCCTATTTCGG-3′ (nucleotides 2054–2073 of M13mp19) were simultaneously annealed to a single-stranded noncoding template of the *lacI*-M13 clone, extended with E. coli DNA polymerase I large fragment (Klenow), and ligated essentially as described by Zoller and Smith (1984). Following transformation into strain JM101 and screening for the mutant template, single-stranded DNA of the mutated *lacI* (*lacI*^{m})–M13 was prepared, and the

entire DNA insert was sequenced by the dideoxy chain-terminating method (Sanger et al., 1977; Biggin et al., 1983). Double-stranded (M13 Rf form) DNA for the mutant *lacI*^{m} gene was excised with EcoRI and HincII and was reintroduced into the corresponding sites of an SP62 vector together with the 0.8 kb HincII–EcoRI DNA fragment of the 3′ portion of the *lacI* gene. This mutant plasmid was designated pI^{m}SP62. To delete the operator sequence downstream of the termination codon of the *lacI* gene, pI^{m}SP62 DNA was digested with BamHI and SphI, thus generating a 3′-protruding end that protected the remainder of the vector from exonuclease III attack, and unidirectionally deleted by exonuclease III (Henikoff, 1984). This DNA was rendered blunt-ended with T4 DNA polymerase, ligated with T4 DNA ligase, and used to transform the MC1061.2 strain of E. coli. Subsequently, these plasmids were excised by EcoRI and SalI digestions, and the mutant *lacI*^{m}-containing fragments were cloned into the corresponding sites of the M13mp18 vector. Single-stranded DNA was prepared from a plaque-purified phage, and the DNA sequence at the deletion junction was determined using the dideoxy chain-terminating method (Sanger et al., 1977; Biggin et al., 1983).

Construction of the *lacI* Expression Vector pRSV-I

The plasmid pI^{m}SP62, carrying the entire sequence of the *lacI* gene (from 30 nucleotides upstream of the primary translation initiation site to 18 nucleotides downstream of the termination codon) was digested with EcoRI, blunt-ended with Klenow fragment in the presence of dNTPs, annealed with HindIII linker, ligated with T4 DNA ligase, and digested with HindIII. The 1.3 kb HindIII–SalI fragment was purified by electrophoresis in low-gelling-temperature agarose (1.0% SeaPlaque agarose; FMC Corporation, Marine Colloids Division) and was subcloned into pRSV-CAT (Gorman et al., 1982b). This plasmid was digested with HpaI, annealed with SalI linker, ligated with T4 DNA ligase, and then redigested with SalI and HindIII. The 3.5 kb HindIII–SalI fragment was gel-isolated and then ligated with the 1.3 kb HindIII–SalI fragment of *lacI*^{m} and then used to transform MC1061.2. In the resulting plasmid, designated pRSV-I, a *lacI*-encoding region is installed under transcriptional control of the RSV LTR. This plasmid carried an SV40 poly(A) addition signal and was used for the expression of the *lac* repressor protein in LTK$^-$ cells (see below).

Construction of CAT Plasmids Containing Operator Inserts

Plasmid pOE101, carrying 12 tandem copies of the *lac* operator (Sadler et al., 1980), was partially digested with EcoRI. The ends of the operator monomer and oligomers were then repaired with Klenow fragment in the presence of dNTPs. Operator-inserted CAT plasmids were constructed by the following procedures. First, for insertion between the initiation codon (ATG) and the transcription start point, parental plasmids pSM11 and pSM12 (containing the MSV enhancer in the opposite orientations, respectively, and also containing the SV40 21 bp repeats and its promoter, the coding sequences from the CAT gene, and the SV40 poly[A] addition signal; Laimins et al., 1984) were linearized by digestion with StuI and HindIII. The DNA molecules were fractionated by agarose gel electrophoresis, and the linearized large fragments (about 6 kb) of the plasmids were extracted and purified. The ends of the DNA fragments were repaired with Klenow fragment in the presence of dNTPs, and the blunt-ended operator monomer and oligomers mixture was then added and ligated with T4 DNA ligase. Thus, the StuI–HindIII segment (17 bp) of each parental plasmid was replaced by single or multiple tandem operator sequences in the resultant plasmids. Second, for insertion between the transcription start point and the TATA box region, pSM11 and pSM12 was digested with BglI, repaired with Klenow fragment in the presence of dNTPs, incubated with the blunt-ended operator monomer and oligomer mixture, and simultaneously ligated with T4 DNA ligase and used to transform MC1061.2. The resultant plasmids thus contained single or multiple tandem operator sequences between the transcription start and the TATA box regions. Third, for insertion between the TATA box and SV40 21 bp repeats regions, pSM11 or pSM12 was partially digested with NcoI, and the full-length linearized plasmid DNA was fractionated by agarose gel electrophoresis. The ends of the DNA were filled in with Klenow fragment in the presence of dNTPs, the blunt-ended operator monomer and oligomers were added, and the DNA mixture was ligated with T4 DNA ligase. The resultant plasmids therefore included single or multiple tandem operator sequences between the TATA box and the SV40 21 bp repeat regions. Subsequently, all of the operator-containing plasmids were excised by EcoRI and SalI digestion and were cloned into the corresponding sites of M13mp18. That single or multiple tandem operator sequences were in fact introduced into the CAT plasmids at the sites indicated was verified by DNA sequencing (Sanger et al., 1977; Biggin et al., 1983).

Cell Culture and Stable Transfectants

Mouse LTK$^-$ cells were grown in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal calf serum. Cells to be transfected were plated on the day before transfection at a density of 1.0×10^6 cells per 60 mM dish. LTK$^-$ cells were cotransfected with a 50:1 molar ratio of pRSV-I and pTK-5 (Roach et al., 1984), which carries the 3.5-kb *tk*-coding BamHI fragment of the herpes simplex virus type I *tk* gene. Calcium phosphate precipitates of the plasmid DNA were prepared as described elsewhere (Gorman et al., 1982a). The precipitate was left on the cells for 16 hr, a fresh aliquot of medium was supplied, and the cells were incubated without selection for 24 hr prior to addition of HAT medium (DME medium containing 10% fetal calf serum, 0.1 mM hypoxanthine, 0.4 μM aminopterin, 16.0 μM thymidine). Resistant colonies were isolated and expanded after 14 days. Transfectants expressing the *lacI* gene were initially identified by RNA dot blots and were then characterized by an operator-binding assay to quantitate the amount of functional repressor protein (see below). In addition, the levels of repressor protein in the nucleus and in the cytoplasm were quantitated and estimated by Western blotting and radiographic detection with antibody.

Transient Transfection and CAT Assay

LTK$^-$ cells and *lac* repressor–producing cells (LI-1) were grown in DME supplemented with 10% fetal calf serum and in HAT medium, respectively. Cells to be transfected were plated on the day prior to transfection at a density of 1.2×10^6 cells per 60 mM dish. DEAE-dextran transfections were performed by washing the cells twice with DME without serum and then adding 500 μg/ml DEAE-dextran (Sigma Chemical Co., MW 500,000) in DME with the appropriate DNA in the presence of chloroquine (2 μg/ml). Incubations were for 4 hr at 37°C including 1 hr of initial incubation in 2% CO_2 and 3 hr of incubation in 5% CO_2 as described by Sussman and Milman (1984). The cells were shocked with dimethyl sulfoxide for 2 min at 37°C according to Sussman and Milman (1984); the media were replaced, either with or without IPTG (20 mM final concentration), and the cells were incubated for 48 hr before harvesting. Each plasmid preparation used for transfection was purified through two CsCl-ethidium bromide equilibrium gradients. After transfection, the CAT extractions and assay were performed using equal amounts of protein from the cellular extracts according to Gorman et al. (1982a).

Isolation and Analysis of Plasmid DNA from Transfected Cells

Total DNA was isolated from nuclei from transfected cells by the method of Hirt (1967). The supernatants were phenol-extracted twice, chloroform-extracted once, and the DNA was ethanol-precipitated. The pellets were washed with 75% ethanol, dried, and dissolved in 20 μl of TE (10 mM Tris–HCl, 1 mM EDTA). Equivalent portions of each sample were digested with BamHI. The digests and an equivalent portion of each undigested sample were electrophoresed on 1% agarose gels and then blotted to Zeta-probe (Bio-Rad) or Hybond-N (Amersham) membranes, and plasmid DNA was visualized by hybridization with ^{32}P-labeled SP6-CAT RNA. After autoradiography, the resultant bands were quantitated by densitometric scanning and comparison with the intensities of bands of known amounts of purified plasmid DNA.

Isolation and Analysis of RNA from Transfected Cells

Cytoplasmic RNA was isolated from mouse LTK$^-$ or LI-1 cells. Transfected cells were washed with PBS (phosphate-buffered saline), scraped off with 5 ml of PBS, and centrifuged. The pellet was resuspended in 0.1 ml of 0.25 M Tris–HCl, and the cell suspension was frozen and quickly rethawed four times on dry ice. Nuclei were removed by centrifugation in an Eppendorf microfuge for 15 min (4°C), and RNA was isolated from the resulting supernatant according to Gorman et al. (1982b). An equal volume of a solution of 7 M urea, 0.35 M NaCl, 10 mM Tris–HCl (pH 8.0), 10 mM EDTA, 1% SDS and about 40 μg of tRNA per ml were added. The mixture was extracted twice with

phenol–chloroform–isoamyl alcohol (20:20:1 [v/v]) and twice with chloroform–isoamyl alcohol. After precipitation with ethanol the RNA was collected by centrifugation and was resuspended in 10 mM Tris–HCl, 0.1 mM EDTA, 0.1% SDS. Equivalent portions of each RNA sample were assayed by dot-blot analysis using an SP6-generated antisense *lacI* probe, an SP6-generated antisense CAT probe, or an SP6-generated antisense actin probe (Hu et al., 1986). Approximately 70 μg of each cytoplasmic RNA preparation was used for each primer extension experiment, and the 36 nucleotide ^{32}P-labeled oligonucleotide 5′-TGCCATTGGGATATATCAACGGTGGTATATCCAGTG-3′ (complementary to a region of the CAT mRNA started at 12 nucleotides downstream of the initiation codon AUG) was used as primer. Primer extension was performed as previously described (Hu et al., 1986) except that the cloned Moloney murine leukemia virus reverse transcriptase (BRL) was used in extension.

Western Blot Analysis

Crude cytoplasmic and nuclear extracts from LI-1 and LTK$^-$ cells were prepared from two 100 mM dishes near confluence. The cells were washed with PBS, scraped off with 5 ml of PBS, and centrifuged. The pellet was resuspended in 0.1 ml of cold lysis buffer containing 0.2 M potassium phosphate (K_2HPO_4:KH_2PO_4 = 5:1, pH 7.4), 0.1 mM EDTA, 0.3 mM DTT, 5% (w/v) glucose, 25 μM leupeptin, 1 μg/ml bestatin, 10 μg/ml trypsin inhibitor, and 1 mM PMSF. The cell suspension was freeze-thawed four times on dry ice, and the nuclei and the cell debris were sedimented. The supernatant was used for Western blot analysis and for the repressor–operator filter-binding assay (see below). The nuclear pellet was resuspended in 0.1 ml of homogenization buffer (0.5% NP40, 0.2 mM EDTA) and homogenized. The homogenate was treated with DNAase I (1 mg/ml final concentration) for 1 hr at 37°C and then mixed with the electrophoresis sample solution (2× ESS: 20 mM Tris–HCl, 5 mM EDTA, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.5 mg/ml bromophenol blue [pH 7.0]). Equal amounts of protein from cytoplasmic extracts of LI-1 and LTK$^-$ cells (~2 × 10^6) were also mixed with equal volumes of 2× ESS. In addition, ^{14}C-labeled molecular weight standards (BRL) were included. The samples were subjected to SDS–10% polyacrylamide gel electrophoresis and then transferred to nitrocellulose according to Burnette (1981). The filter was probed with anti-repressor monoclonal antibody B-2 (Sams et al., 1985) followed by ^{125}I-labeled sheep anti-mouse immunoglobulin (Amersham), air-dried, and exposed to X-ray film. The amount of repressor protein was quantitated by scanning the autoradiograph.

Other Methods

Nucleic acid manipulations and blot hybridizations were according to standard procedures (Maniatis et al., 1982; Reed and Mann, 1985). In vitro transcription with SP6 polymerase employed methods and reagents as described by Melton et al. (1984). The filter-binding assay of repressor–operator complexes was performed essentially as described by Lin and Riggs (1972) except that the DNA probe used was a gel-purified ^{32}P-end-labeled operator fragment of 40 bp. Protein content of cellular extracts was measured by the method of Bradford (1976).

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