Cytochemical observation of regulated bacterial β -galactosidase gene expression in mammalian cells

(repressor/gene regulation/transfection/*lacI*/*lacZ*)

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ABSTRACT Bacterial β -galactosidase, encoded by the *lacZ* gene, serves as a sensitive cytochemical marker in eukaryotic cells and tissues. In transient expression experiments, human and simian cells stain blue 48 hr after transfection with a plasmid containing a *lacZ* gene, whose expression is directed by a simian virus 40 promoter containing a synthetic lactose operator sequence. Transfection efficiency was about 0.6%. Incorporation of an operator sequence within the promoter permits regulation of β -galactosidase gene expression by the *lacI* gene product, the *lac* repressor. When cells were cotransfected with the *lacZ* plasmid and a second plasmid containing the *lacI* gene, β -galactosidase activity was extinguished. Its activity could be reestablished to original levels upon application of isopropyl β -D-thiogalactoside to transfected cells. A cell line that stably carries both the *lacI* and *lacZ* genes was efficiently induced to synthesize β -galactosidase after isopropyl β -D-thiogalactoside administration. In transient expression experiments and in stably transfected lines, repression and induction of β -galactosidase activity were predominantly at the transcriptional level.

The ability to dictate the reversible expression and repression of specific eukaryotic genes represents a potent tool for studying cellular activity. Efforts to this end have primarily utilized the mouse mammary tumor virus promoter or the mouse metallothionein promoter fused to a heterologous gene or cDNA (1-7). In the former case, administration of glucocorticoid, which binds a glucocorticoid receptor, results in gene activation. The ligand-receptor complex interacts with an enhancer-like glucocorticoid receptor element in the mouse mammary tumor virus promoter, thereby stimulating transcriptional activity. In the latter case, gene activation is induced by exposure to heavy metals. Although both systems have been used with variable success, they are limited by restrictions in tissue-type expression and in substantial background levels of expression in cell types that permit induction. Other inducible promoter systems that have been used to achieve regulated gene expression, but with similar reservations, include heat-shock-inducible promoters (8, 9) and poly(IC)-inducible promoters (10).

The concept of using bacterial regulatory systems in mammalian cells has numerous attractive features. These systems have the potential for imposing very stringent regulation in a tissue-independent manner. There is also a reduced likelihood that prokaryotic regulatory proteins will interact with host DNA sequences that are similar to target sequences, in a biologically meaningful fashion. One concern in adapting a prokaryotic regulatory system to a mammalian nuclear milieu is whether or not prokaryotic regulatory proteins can access DNA in chromatin efficiently and with specificity. This concern is relieved by the demonstration that the *cre-lox*

site-specific recombination system of coliphage P1 can operate efficiently in yeast (11). The *cre* recombinase is able to enter the nucleus and to mediate precise recombination events within eukaryotic chromosomes. Furthermore, the *Escherichia coli* *lexA* operator and repressor are able to effect regulation of gene expression in yeast. More recently, the *E. coli* lactose regulatory system has been used to control expression of the chloramphenicol acetyltransferase (CAT) gene in simian and rodent cells (12-14), indicating that prokaryotic regulators can function in mammalian cells.

Expression of the bacterial β -galactosidase gene has proven a convenient and sensitive biochemical (15-17) or cytochemical (16-22) measure of specific gene activity in cultured mammalian cells (16) and in transgenic (18-21) and chimeric animals (22). Thus, cytochemical detection of β -galactosidase activity should provide a useful means for visualizing the regulation of gene expression at the cellular level. In this report, we have taken advantage of the *E. coli* lactose regulatory system, which can function in mammalian cells (12-14), to control the expression of a bacterial β -galactosidase gene in human and simian cells and to cytochemically observe the repression and induction of β -galactosidase activity in individual cells.

MATERIALS AND METHODS

Cell Culture and DNA Transfection. Human HTD114 cells (23) and simian CV-1P cells (provided by James Figge, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA) were maintained at 37°C in 5% CO₂/95% air. The HTD114 cells, an APRT⁻ derivative of HT1080, were cultured in minimal essential medium (MEM) plus nonessential amino acids supplemented with 7.5% (vol/vol) horse serum (Hyclone) and 2.5% (vol/vol) fetal calf serum (GIBCO). The CV-1P cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum.

For transient expression, cells were plated at 1.4×10^4 cells per cm² in either 60-mm or 100-mm plates as described (24). Supercoiled plasmid DNA was introduced into cells by calcium phosphate coprecipitation (25) and 4 hr later cells received a glycerol shock (26). After transfection, cells were either fixed and stained for β -galactosidase activity (21) or, after 48 hr, collected, lysed, and pelleted to assess the cleared lysates for β -galactosidase activity (16) or CAT activity (27). Isopropyl β -D-thiogalactoside (IPTG) induction of β -galactosidase entailed a 48- or 72-hr cell exposure to 20 mM IPTG unless otherwise indicated.

Plasmid Construction. Plasmids pCMVlacI and pSVlacO-CAT were generously provided by James Figge (13, 14). The *lacZ* gene contained in the final construct pSVlacOZ (21) was a kind gift from Lap-Chee Tsui (University of Toronto, Toronto, ON). The *lacZ* gene with attendant simian virus 40

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Abbreviations: IPTG, isopropyl β -D-thiogalactoside; CAT, chloramphenicol acetyltransferase.

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polyadenylation signal was inserted into Bluescript (Stratagene) as a 3.7-kilobase (kb) *Hind*III–*Bam*HI fragment in which the *Bam*HI site had been blunt-ended and ligated to a blunt-ended *Xho*I site in the Bluescript polylinker. This strategy regenerates the *Xho*I site and produces the plasmid pLZ6.6. A simian virus 40 promoter with a synthetic *lac* operator located between the “TATA box” and the transcription start site was derived as a 1.1-kb *Hind*III fragment of pSVlacOCAT and inserted into the unique *Hind*III site of pLZ6.6 upstream of the *lacZ* gene to produce the plasmid pSVlacOZ. The orientation of the promoter was established by the relative positions of asymmetric *Eco*RV sites.

RNA Detection. Northern blot analysis was performed as described by Thomas (28) by using 20 μ g of total RNA fractionated in a 1.2% agarose gel containing 2.2 M formaldehyde that was electrophoresed for 4 hr at 75 V. The hybridization probe was a 0.4-kb *Eco*RI–*Acc*I fragment of the *lacZ* gene that was labeled by random priming by using [α - 32 P]dATP. Ribonuclease protection assays utilized an [α - 32 P]UTP-labeled *in vitro* transcript (29) of the same *Eco*RI–*Acc*I *lacZ* fragment cloned into Bluescript. The labeled RNA was hybridized with 12 μ g of total RNA for 16 hr at 53°C in 15 μ l of buffer containing 80% (vol/vol) deionized formamide, 0.4 M NaCl, 1 mM EDTA, and 40 mM Mops (pH 7.0). After RNase A digestion, the sample was fractionated on a 5% polyacrylamide gel at 150 V for 3 hr. The gel was dried and exposed to Kodak X-AR5 film.

RESULTS

Human HTD114 cells transfected with pSVlacOZ, manifest cytochemically detectable β -galactosidase activity 48 hr after transfection (Fig. 1*a*). After cotransfection with pSVlacOZ

and pCMVlacI, the number of stained cells was reduced by a factor of at least 30, resulting in only 1 of 5000 cells examined staining positive for bacterial β -galactosidase (Fig. 1*b* and Table 1). Administration of IPTG prior to and during cotransfection reestablished β -galactosidase expression to the same level as that obtained by transfection with pSVlacOZ alone (Fig. 1*c* and Table 1).

To determine whether other cell lines would support regulation of the β -galactosidase gene in pSVlacOZ by the *lac* repressor, the simian cell line CV-1P was tested and found to be as effective as HTD114 cells (data not shown). To assess the relative level of *lacI* gene needed to suppress expression of the *lacZ* gene in these cell lines, HTD114 and CV-1P cells were transfected with a constant amount of pSVlacOZ DNA and increasing amounts of pCMVlacI. Cleared cell lysates were assayed for β -galactosidase activity 48 hr after transfection or cotransfection. At a pCMVlacI/pSVlacOZ molar ratio of about 0.05, β -galactosidase expression was reduced by greater than 75% compared with that seen with pSVlacOZ transfection alone. At molar ratios of 0.4 and greater, β -galactosidase expression was reduced to background levels (Fig. 2).

The capacity for mammalian cells to support β -galactosidase induction by IPTG was tested by both transient and stable transfections. In the transient expression experiments, human HTD114 cells and simian CV-1P cells were transfected with pSVlacOZ alone or concomitantly with pCMVlacI, with or without administration of IPTG. As shown in Fig. 3, administration of IPTG supported reestablishment of β -galactosidase activity to a level equal to that obtained by transfection with pSVlacOZ alone. To establish stable trans- fected lines carrying both plasmid DNAs, Apr^r HTD114

FIG. 1. Visualization of β -galactosidase repression and induction in HTD114 cells. Approximately 10^5 human HTD114 cells were seeded into each 35-mm well of a 6-well culture dish. Twenty-four hours later the cells were transfected with pSVlacOZ alone or cotransfected with pCMVlacI. Forty-eight hours after transfection, cells were stained for β -galactosidase activity. (*a*) Transfection with 4 μ g of pSVlacOZ + 26 μ g of Bluescript. (*b*) Cotransfection with 4 μ g of pSVlacOZ and 26 μ g of pCMVlacI. A single stained cell (arrow) was visualized. (*c*) The same cotransfection as in *b* but cells were exposed to IPTG for 24 hr before staining. (*d*) Transfection of cells with 30 μ g of the Bluescript vector alone.

Table 1. Induction and repression of β -galactosidase expression in HTD114 cells

Transfecting DNA(s)	DNA added, μ g	Cells stained, %
pSVlacOZ	4	0.64
+ Bluescript	26	
pSVlacOZ	4	0.02
+ pCMVlacI	26	
pSVlacOZ	4	0.88
+ pCMVlacI	26	
+ 20 mM IPTG		
Bluescript	30	0

Cells in 35-mm wells were transfected with pSVlacOZ alone or with pCMVlacI. After 48 hr, cells were stained and the percent of stained cells was determined. The percentages were based on a total of five separate counts of 1000 cells in randomly selected fields from two separate experiments in each case (thus a total of 5000 cells was counted).

cells were first cotransfected with pSVlacOZ and ptkneo, and G418-resistant cells were selected. Cells from individual colonies were also tested for β -galactosidase activity by cytochemical staining. Positive colonies were subsequently cotransfected with pCMVlacI and plasmid pSAM4.4, which contains the mouse *aprt* gene. Cotransfected cells were selected in AAA medium (23), which selects for cells that are *APRT*⁺, and individual colonies were examined for levels of β -galactosidase activity. Clone HSL2 was selected and expanded. When tested for β -galactosidase activity, the cells exhibited little stain. However, after exposure to IPTG, the cells were readily stained for β -galactosidase (Fig. 4), indicative of efficient induction.

The transcriptional status of the *lacZ* gene was assessed in the presence or absence of repressor and of IPTG. Cells transfected with pSVlacOZ produced a 3.2-kb band by Northern blot analysis in a transient expression experiment (Fig. 5A, lane a). When cotransfected with pSVlacOZ and pCMVlacI, the RNA band was diminished by a factor of about 10 but remained detectable (Fig. 5A, lane c). Admin-

FIG. 2. Level of *lacI* gene needed for β -galactosidase repression. Approximately 10^6 HTD114 cells were cotransfected with 7.7 μ g of pSVlacOZ and various amounts of pCMVlacI. After 48 hr, the cells were collected and lysed and the cleared lysate was assayed spectrophotometrically for β -galactosidase activity [shown in microunits/ μ g (μ U/ μ g)]. All transfections contained 20 μ g of plasmid DNA, using appropriate amounts of Bluescript DNA to bring DNA levels to 20 μ g. The molar ratio of pCMVlacI DNA to pSVlacOZ DNA is in parentheses. Bars: 1, pSVlacOZ; 2, pSVlacOZ + 0.24 μ g of pCMVlacI (0.05); 3, pSVlacOZ + 0.72 μ g of pCMVlacI (0.14); 4, pSVlacOZ + 2.2 μ g of pCMVlacI (0.44); 5, pSVlacOZ + 3.8 μ g of pCMVlacI (0.76).

FIG. 3. Induction of β -galactosidase production by IPTG. Approximately 10^6 HTD114 cells were seeded in 100-mm dishes and 24 hr later cells were transfected with 7.7 μ g of pSVlacOZ and 12.3 μ g of Bluescript DNA as carrier. Alternatively, they were cotransfected with 7.7 μ g of pSVlacOZ and 7.7 μ g of pCMVlacI. After 48 hr, cells were collected with a rubber policeman and cleared lysates were assayed for β -galactosidase activity [shown as microunits/ μ l (μ U/ μ g)]. Bars: 1, 7.7 μ g of pSVlacOZ + 7.7 μ g of pCMVlacI; 2, 7.7 μ g of pSVlacOZ + 7.7 μ g of pCMVlacI + 20 mM IPTG added 24 hr prior to transfection; 3, 7.7 μ g of pSVlacOZ + 12.3 μ g of Bluescript DNA; 4, 20 μ g of Bluescript DNA.

istration of IPTG prior to and during transfection reestablished the *lacZ* transcript to its original level (Fig. 5A, lane b). Similar results were obtained with the cell line HSL2, which carries the *lacI* and *lacZ* genes as stable integrants. In the absence of inducer, the *lacZ* mRNA is barely perceptible (Fig. 5B, lane a) but is more apparent upon overexposure. After IPTG administration, *lacZ* RNA is detectable at about a 10-fold increased level (Fig. 5B, lane b). Ribonuclease protection after solution hybridization experiments provides confirmatory results (Fig. 5C).

DISCUSSION

The experiments reported here describe a sensitive and rapid cytochemical approach that permits visual assessment of the transcriptional status of a reporter gene, its repression by the *lac* repressor and its induction by IPTG. The staining techniques utilize standard procedures for detecting the presence or absence of β -galactosidase activity. The efficacy of *lac* repressor function is apparent in both transient and stable transfections, and cytochemical results were confirmed by biochemical analyses of enzyme activity. The promoter used

FIG. 4. Induction of β -galactosidase production in a stably transfected cell line. Approximately 10^5 HSL2 cells, which are HTD114 cells containing stable integrants of pSVlacOZ and pCMVlacI, were seeded into 35-mm wells and stained for β -galactosidase activity in the absence of IPTG (a) and after a 48-hr exposure to 20 mM IPTG (b).

FIG. 5. Regulation of *lacZ* transcription during transient expression and in a stably transfected cell line. (A) Northern blot of *lacZ* RNA after transfection as follows. Lanes: a, 7.7 μ g of pSVlacOZ + 22.3 μ g of Bluescript; b, 7.7 μ g of pSVlacOZ + 7.7 μ g of pCMVlacI + 14.6 μ g of Bluescript + 20 mM IPTG; c, same as lane b but without IPTG; d, 7.7 μ g of pCMVlacI + 22.3 μ g of Bluescript. (B) Northern blot of *lacZ* RNA produced as follows. Lanes: a, stably transfected HSL2 cells; b, HSL2 cells after a 48-hr exposure to 20 mM IPTG. In A and B the probe was a 0.4-kb *EcoRI*-*Acc* I DNA fragment derived from the *lacZ* gene and radiolabeled by random priming. (C) Protection of a *lacZ* RNA fragment from RNase A digestion. The probe was an antisense transcript of the same 0.4-kb *EcoRI*-*Acc* I fragment of the *lacZ* gene, radiolabeled *in vitro*, and hybridized as follows. Lanes: a, control, sense RNA produced *in vitro* from the same fragment; b, total RNA from uninduced HSL2 cells; c, total RNA from HSL2 cells exposed to 20 mM IPTG for 48 hr. RNA sizes are indicated in kb.

to direct β -galactosidase synthesis is the simian virus 40 promoter containing a synthetic *lacO* analog positioned between the TATA box and transcription start site, the construction of which has been described (13, 14). The *lacI* gene has been modified to contain a eukaryotic ATG translation start codon, and its expression is directed by a cytomegalovirus early gene promoter (13, 14). The *lacI* gene product, the *lac* repressor, is a 38-kDa peptide that contains 360 amino acid residues and that comprises the four subunits of a homotetramer (30, 31) that binds tightly to the *lac* operator sequence and impedes transcription. Consistent with earlier reports, the subunits must be capable of associating into a tetramer within a mammalian cell milieu, and must be stable enough to localize in the nucleus at a concentration sufficient to exert its repressive effect. A model proposed by Lin and Riggs (32, 33) predicts that about 1500 repressor molecules would be required to effect a 99% repression from two operator-containing transcription units in a eukaryotic nucleus. Based on immunological data, Hu and Davidson (12) have estimated that nuclei of L cells transfected with a *lacI* expression vector contain about 2500 repressor molecules, which would be sufficient to repress the *lacZ* gene.

After cotransfection with pSVlacOZ and pCMVlacI, repression of β -galactosidase activity can be rendered virtually complete. At a pCMVlacI/pSVlacOZ molar ratio of 0.05, enzyme activity was reduced almost 80%; at a ratio of less than 0.4, enzyme activity was at background levels, characteristic of cells transfected with Bluescript vector alone. In contrast to these results, an earlier report using CAT gene expression as a marker in CV-1P cells, indicated that about a 10:1 pCMVlacI/pSVlacOZ ratio was required to effect extinction of CAT activity (13). We have performed similar experiments by cotransfecting pSVlacOZ and pCMVlacI into CV-1P cells, with results consistent to those obtained with pSVlacOZ. The sensitivity of β -galactosidase detection and CAT detection was similar (data not shown). The basis for the incongruity is unclear.

In the cell lines used in this study, a cytomegalovirus early promoter directs *lacI* expression at a high enough level to repress reporter genes directed by a simian virus 40 promoter containing a modified operator sequence, even when the former promoter is present at a submolar ratio. However, since relative promoter strengths vary in different cells, it would be useful to develop approaches that increase the efficiency by which genes under *lac* regulation are repressed. In mammalian cells, about 10% of total *lac* repressor appears to be located in the nucleus, with similar repressor concentrations in the nucleus and the cytoplasm (12). This observation suggests that the repressor enters the nucleus passively and that the small percentage of molecules in the nucleus exerts the phenotypic effect. The *lac* repressor appears to contain no obvious nuclear localization signal (31), which is consistent with its apparently random cellular distribution. In principle, one of several known nuclear localization signals (34) could be appended to the repressor molecule to increase the proportion of molecules available for repressor function. Appending a short peptide with nuclear localization activity would require that it not interfere with homotetramer formation, operator DNA binding, or effector binding. An exhaustive mutagenesis study of the *lacI* gene indicates that the carboxyl-terminal region of the repressor would be the least obtrusive site for such an insertion (35). Efficiency of repression can also potentially be enhanced by adding a second operator sequence to the promoter region of the gene under repressor regulation (12). Including two operator sequences, optimally spaced by about 150–300 base pairs, contributes to possible DNA loop generation in the presence of repressor (36, 37), rendering repression of transcription more efficient.

To have biological utility, repression by the *lac* repressor must be reversible. The gratuitous inducer, IPTG, is an allosteric effector that binds the *lac* repressor, reducing its affinity for the operator DNA sequence by a factor of >300 (38). Expression of the repressed gene is thereby reestablished. Administration of IPTG to cells cotransfected with plasmids pSVlacOZ and pCMVlacI yields similar numbers of stained cells and similar levels of β -galactosidase activity as produced by transfection with pSVlacOZ alone. Likewise, administration of IPTG to cells stably transfected with pSVlacOZ and pCMVlacI DNAs reestablishes β -galactosidase activity. The availability of a stringently regulated genetic system in mammalian cells that can be activated by an inducer is highly desirable. The *E. coli* lactose regulatory system serves as an informative paradigm and would allow development of alternative strategies for analysis of cellular activities. For example, deleterious, dominant-acting genes under control of the *lac* repressor could be introduced into cells without disturbing the normal cellular phenotype. Administration of IPTG, the gratuitous inducer, would permit activation of the deleterious activity so that its effect on cellular phenotype could be analyzed in a temporal fashion. In principal, this technology can be extended to transgenic animals. This approach would afford an *in vivo* strategy for reversibly turning genes on and off that encode products deleterious to cells in a tissue-specific manner. It also has the potential for providing information regarding cell lineage relationships and for applications relating to *in vivo* mutagenesis.

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