ABSTRACT

We have studied the lac repressor (lacR) system in two breast cancer cell lines, MCF-7 and MDA-MB-231, in vitro and in vivo. Breast cancer cell lines were stably transfected with lacR and tested for inducibility by transient transfection with a lac operator/luciferase reporter plasmid. The level of expression of lacR did not appear to correlate with the basal or maximal activation of induction by isopropyl β-D-thiogalactoside (IPTG). Stable transfection with the same reporter gene resulted in up to 40-fold (MDA-MB-231) and 50-fold (MCF7) induction. In the absence of IPTG, a low level of basal reporter gene expression was seen in all clones. Detailed analysis showed that induction was rapid (maximal at 24 h), reversible (a return to basal expression by 24 h) and dose-dependent. To test if this system was also inducible in vivo, tumors were biopsied at several time points following administration. IPTG caused a 10-fold increase in luciferase activity after 8 h, which persisted for 24 h. Thus, this system allows tightly controlled inducible in vivo and in vitro gene expression with low basal expression, and it may provide an important tool for the study of lethal genes in human breast cancer cells.

INTRODUCTION

There are several systems for controlling gene induction in mammalian cells. Naturally occurring promoters that are themselves inducible by external stimuli have been used; for example, metallothionein promoter (15), heat-shock promoters (13) and mouse mammary tumor virus promoter (16). The pitfall with these systems is that they are generally “leaky” under non-induced conditions, have only small to modest levels of induction and the inducers of these promoters have biologic effects within the cells. Two recently reported inducible systems, the tetracycline (6) and edcylone (11) expression systems, show high levels of induction in mammalian cells and animals. While both systems allow induction of up to 1000-fold, both can have high basal expression, which is undesirable for expression of toxic or growth-inhibitory genes.

The lac repressor system has been developed from the prokaryotic lac operon (2), where lac repressor (lacR) binds as a homotetramer to a sequence called the lac operator in the promoter of the β-galactosidase (β-gal) gene. Binding of allo lactose, or the synthetic inducer isopropyl β-D-thiogalactoside (IPTG), lowers the affinity of lacR for β-gal promoter (IPTG), lowers the affinity of lacR for operon promoter, thereby allowing transcription. This system of inducible expression has been adapted for mammalian cells (3, 7) and has a number of advantages over other systems. First, there are probably no lac operator sequences within regulatory sequences of eukaryotic promoters. Second, and most important in the control of toxic gene expression, the system involves a relief of repression of transcription rather than an induction of a basal level of transcription, so that basal expression remains low.

Several genes in different cell types have been expressed under the control of the lac repressor system including Ha-ras (10), simian virus 40 (SV40)-T antigen (4), growth hormone (8) and human adrenergic receptor (5). Transgenic animals have been generated that express both lacR and SV40 large T antigen (lacO) under the control of lac operator sequences (4); however, a complete repression of transcription was not seen. Wyborski et al. (17) created transgenic mice expressing both lacR and lac operator/luciferase reporter constructs; however, IPTG caused no increase in reporter gene expression either in the animal or in primary cultured cells.

Here, we show how the lac repressor system can be used in controlling gene expression in two breast cancer cell lines in vitro. Induction is rapid, reversible and dose-responsive. Additionally we have shown that the lac repressor system can be used to control in vivo inducible gene expression in breast cancer grown as a xenograft tumor in nude mice.

MATERIALS AND METHODS

Plasmids

The LacSwitch™ Mammalian Inducible Expression System was purchased from Stratagene (La Jolla, CA, USA). A multiple cloning site (MCS) was introduced into the unique NotI site of pOPRSVCAT by ligating the oligonucleotides listed in Table 1, which thereby remove the chloramphenicol acetyl transferase (cat) gene. The resulting plasmid (pOPRSVMCS) was sequenced to determine orientation of the MCS. pOPRSVLuc was generated by digesting the luciferase gene from pGLBasic with Xho/BamHI and ligating this fragment into the XhoI/BclI sites of pOPRSV.

Cell Culture and Transfection

MCF-7 cells were originally obtained from Dr. C.K. Osborne (12). MDA-MB-231 cells (Clone 10A) were kindly provided by Dr. V.C. Jordan (9). Cells were routinely maintained in improved minimal essential medium (IMEM), 10% fetal bovine serum (FBS), 2 mM glutamine, 50 I.U./mL penicillin and 5 μg/mL streptomycin. For transient and stable transfection, LIPOFECTIN® (Life Technologies, Gaithersburg, MD, USA) was used according to the manufacturer’s instructions. For transient assay, cells were transfected with 0.5 μg of reporter plasmid and 0.2 μg of pSVβ-gal (to correct for transfection efficiency) and then stimulated with 1 mM IPTG (Sigma Chemical, St. Louis, MO, USA) for 24 h. Cells were then lysed and measured for luciferase (Promega, Madison, WI, USA) and β-gal (Galacto-Light™; Tropix, Bedford, MA, USA) activities. For stable transfection, cells were transfected with 30 μg of either p3’SS (eukaryotic LacR expression plasmid) or pOPRSVLuc plasmid (eukaryotic
lac-operator containing Rous sarcoma virus [RSV] promoter upstream of luciferase). Both plasmids contain both the gene of interest and an antibiotic resistance gene, thus we only transfected a single plasmid at each step. Stable transfectants for LacR (p3′SS) were selected in hygromycin (MCF-7, 200 µg/mL; MDA-MB-231, 600 µg/mL) and for luciferase (pOPRSVLuc) in G418S (MCF-7, 600 µg/mL; MDA-MB-231, 1200 µg/mL).

**Western Blotting**

Cells were grown to sub-confluence in 10-cm dishes and lysed with 5% sodium dodecyl sulfate (SDS). DNA was sheared by sonication (2 × 5 s), and protein concentration was determined by the BCA Protein Assay (Pierce Chemical, Rockford, IL, USA). Equal amounts of protein were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoresed onto nitrocellulose overnight at 4°C. After blocking in 5% milk-TBST (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.05% Tween® 20) for 1 h, membrane was washed three times with TBST and then incubated with a 1:1000 dilution of anti-lacR antibody (Stratagene) in 5% milk-TBST. After another three washes with TBST, antibody was detected with a 1:1000 dilution of horseradish peroxidase (HRP)-linked goat anti-rabbit antibody and enhanced chemiluminescence (ECL™; Amersham, Arlington, IL, USA). M15 bacteria (Qiagen, Chatsworth, CA, USA) that express lacR were used as a positive control.

**In Vivo Growth of MDA-rep4luc2 Cells**

MDA-rep4luc2 cells were grown in the mammary fat pad of the nude mouse. Briefly, mice were injected with 2 × 10⁷ cells, and the tumors were allowed to grow to a size of approximately 7 × 7 mm. At time 0, the mice were anesthetized, biopsies of the tumors were taken with a 14-g Precision-Cut needle (Becton Dickinson, Franklin Lakes, NJ, USA) and the core biopsy was frozen immediately in liquid nitrogen. Mice were then injected intraperitoneally with 200 µL IPTG (0.53 mmol). Mice were re-injected after 4 h, and then biopsies were performed at the following time points (8, Table 1. Multiple Cloning Site Inserted into pOPRSV

| 5′ GGCGGCGGGCCCTGATCAGTGATACTGGGTACGTCGCCG 3′ |
| 3′ CGGGCCGGGAGCATGATACTGGGCTTCATGGGCCGG 5′ |
| XmaI/Smal/Apal | BclII | SpeI | Hpal | Xhol | KpnI |

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24, 32, 48 and 72 h). To account for differences within the tumor, several biopsies were taken from the same tumor. At the final time point, mice were sacrificed, and the tumor was separated into convenient sections for assay. Tissue was crushed under liquid nitrogen and then lysed in reporter lysis buffer (Promega). After 5 min on ice, lysate was sonicated (2 × 5 s) and then centrifuged at 14000 × g for 15 min at 4°C. The supernatant was then immediately assayed for luciferase activity, and protein content was measured.

RESULTS

Expression of lacR Repressor and IPTG Induction in Breast Cancer Cells

Two breast cancer cells lines (MCF-7 and MDA-MB-231) were transfected with lacR, and expression was analyzed by Western blotting (Figure 1). Several

Figure 1. Expression of lacR in MCF-7 and MDA-MB-231 cells and inducibility with IPTG. MCF-7 (left panels) and MDA-MB-231 cells (right panels) were transfected with lacR, and expression was assessed by Western blotting. M15 bacteria, that express lacR, were run as a positive control (left lane in each panel). Positive clones were transiently transfected with 0.5 µg of reporter gene pOPRSVlac and 0.2 µg pSVβ-gal. Cells were incubated with (black bars) or without (open bars) IPTG (1 mM) for 24 h, lysed, and luciferase and β-gal activities were determined. Luciferase units were divided by the corresponding β-gal activity and are presented as the average relative luciferase units of triplicate wells ± standard error of the mean (SEM).

Figure 2. Dose response of induction by IPTG. MDArep4lac2 cells were incubated with increasing concentrations of IPTG (0–1 mM) for 24 h, lysed and luciferase activity was determined. Values represent the average of duplicate wells ± SEM. The figure is representative of two independent experiments.
clones expressing varying levels of lacR, (38 kDa) were obtained. MCF-7 control cells contained a cross-reactive species that was not lacR (upper band). LacR positive clones were then transiently transfected with a reporter gene consisting of an RSV promoter containing lac operator sequences upstream of luciferase (pOPRSVLuc). After transfection, cells were incubated in IPTG (1 mM) for 24 h, lysed and measured for luciferase activity. The RSV promoter had a higher level of expression in MCF-7 cells than in MDA-MB-231 (see scale on left axis compared to right axis [Figure 1]). Several clones showed induction by IPTG (MCF-7rep2-2 and MDA-rep4); however, high levels of lacR did not necessarily confer inducibility (MCF-7rep1-1 vs. MCF-7rep1-6). Furthermore, the absolute level of lacR expression did not correlate with the ability to induce the reporter gene (MDA-rep2 vs. MDA-rep4). Stable clones expressing pOPRSVLuc were generated in MDA-MB-231 and MCF-7 cells. Several clones expressing up to 40-fold (MDA-MB-231) and 50-fold (MCF-7) induction were generated (data not shown). All clones had a similar low level of basal expression. We then performed detailed experiments on one of the stable transfectants (MDA-rep4luc) as shown in Figures 2–4.

IPTG Induction is Dose-Responsive

MDA-rep4luc2 cells were incubated with IPTG (1 mM) for increasing lengths of time (0–48 h), lysed and luciferase activity and protein concentration were determined (closed circles, right axis). Conversely, cells were incubated with IPTG (1 mM) for 48 h, IPTG removed from the media, cells lysed at time points thereafter (0–48 h), and luciferase activity and protein concentration were determined (open circles, left axis). Values represent the average of triplicate wells ± SEM. The difference in activity (left scale vs. right scale) represents experimental variation—the figure being representative of three independent experiments.

IPTG Induction is Rapid and Reversible

IPTG caused a rapid induction of luciferase activity in MDA-rep4luc2 cells (Figure 3). Induction was seen as early as 1 h and reached a maximum of 40-fold induction after 24 h. The increase in luciferase activity could be reversed by removing the IPTG from the media. We consistently observed a transient increase in luciferase activity after removal of IPTG; however, this increase only lasted for 4 h, and the decrease in activity was then rapid—returning to basal level after 24 h.

In Vivo Induction of Luciferase Activity

MDA-rep4luc2 cells (2 × 10^7) were injected into the mammary fat pad of nude mice. When the tumor had...
reached a size of $7 \times 7$ mm (after approximately 3 weeks), the tumors were biopsied. Mice were injected intraperitoneally at 1 and 4 h with IPTG (0.53 mmol), and luciferase activity was measured by biopsying the tumors at time points thereafter. IPTG caused a rapid induction in luciferase activity—reaching 10-fold after 24 h (Figure 4). Interestingly, activity higher than baseline was still detectable up to 72 h after IPTG injection. As luciferase protein has a short half-life of 3–4 h (14), this presumably represents continued luciferase gene transcription.

**DISCUSSION**

Tightly controlled inducible gene expression provides a mechanism for the study of toxic or growth-inhibitory genes in cancer cells. This paper describes the introduction and characterization of the lac repressor system into two breast cancer cell lines (MCF-7 and MDA-MB-231). The system provides a high level of rapid and reversible gene induction, with low basal expression provided by a bacterial protein, lacR. Compared with the tetracycline or ecdysone inducible systems, the lac-switch system is fairly simple. Only a single protein needs to be expressed (lacR) to confer inducibility. However, it is clear from our data that simple expression of lacR does not necessarily confer inducibility. This indicates the importance of first transfecting lacR and analyzing clones before stable transfection with the lac operator plasmid. Simple double transfection with both plasmids will generate a number of clones that fail to show inducibility even though they express lacR. Although expression and targeting to the nucleus has been shown to inhibit transcription of genes engineered with lac operator sequences in the promoter (3), our transfection studies suggest that localization or posttranslational modification of lacR may be important in its ability to cause repression.

One of the major advantages of this system is the ability to produce cells with low levels of basal expression; however, in our breast cancer cells, the absolute values of luciferase activity and mRNA expression (data not shown) were low. This is probably due to low activity of the RSV promoter compared with other promoters (cytomegalovirus [CMV]) or SV40). The RSV promoter may be substituted with a more active promoter; however, we do not know at present if this will affect the resulting basal activity.

In vivo applications of the lac repressor system include gene therapy, antisense mRNA expression, overexpression of toxic or teratogenic genes, etc. One of the advantages of the lac re-
Reduction in gene expression can long the relief of repression can be attained. It is not clear how long the relief of repression can be achieved. In vivo, it is not clear how long the relief of repression can be maintained.

While 72 h post-induction, IPTG was rapidly removed from the circulation (half-life of IPTG is 3–4 h). Ongoing expression of luciferase was observed in vitro for up to 72 h. As luciferase protein has a half-life of 3–4 h, the ongoing expression of luciferase was seen up to 72 h. This suggests that even though IPTG is rapidly removed from the circulation, it may affect the tumor cells for a longer period of time. While 72 h of expression may be sufficient to study some genes in vivo, it is not clear how long the relief of repression can be maintained.

Thus, the system allows for essential nil levels of gene expression in the absence of inducer. Both in vitro and in vivo gene induction, albeit at low levels, can be achieved with IPTG.

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