IPTG-Inducible Episomal Expression System for Exogenous Genes in Primate Cells

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ABSTRACT

An isopropyl β -D-thiogalactopyranoside (IPTG)-inducible episomal expression system has been established for the human breast carcinoma cell line MCF7. This two-component system includes: (i) a primate cell-specific episomal vector, pEpiLac, that contains an IPTG-inducible promoter and (ii) a cell line derived from MCF7, MCF7/LAP5, which expresses the IPTG-dependent transactivator LAP267. Treatment of MCF7/LAP5 cells with IPTG results in efficient inducible expression of exogenous genes from the inducible promoter in pEpiLac. Up to 300-fold induction can be observed when luciferase is used as a reporter. Inducible expression of the p27^{KIP1} cyclin-dependent kinase (CDK) inhibitor, the orphan nuclear receptor Nur77 and the angiogenic inducer Cyr61 has also been demonstrated. Expression of the exogenous gene is promptly halted on removal of IPTG. Moreover, the episomal vector can be stably maintained in and easily recovered from MCF7/LAP5 cells. Taken together, this inducible expression system should be applicable for the regulated expression of exogenous genes, especially growth inhibitory or cytotoxic genes, in cells of primate origin.

INTRODUCTION

The ability to express an exogenous gene in an inducible manner in mammalian cells is often desirable, especially when the gene product is potentially cytotoxic or growth-inhibitory. To this end, several inducible expression systems have been established. Among them is the isopropyl β -D-thiogalactopyranoside (IPTG)-inducible expression system, which consists of an expression vector with a basal promoter linked to the *Escherichia coli lac* operator sequences (3). Genes cloned under the control of this inducible promoter can be specifically activated by the IPTG-dependent transactivator LAP-267, a chimeric protein containing the herpes simplex virus virion protein 16 (VP16) transactivation domain linked to the *E. coli lac* repressor (3). LAP267 functions as a temperature-sensitive transactivator, being active at 32°C but inactive at the normal cell growth temperature of 37°C (3). However, its activity is restored by the presence of IPTG at the nonpermissive temperature (3).

Recently, this IPTG-inducible expression system has been modified, and its background level of expression was reduced (17). A modified mouse mammary tumor virus (MMTV) basal promoter was put in place of the simian virus 40 (SV40) early promoter used in the original vector. Two Oct-1 binding sites within the basal MMTV promoter were destroyed by mutation, resulting in very low basal or leaky expression from the inducible promoter (15). This vector system is efficacious for inducible gene expression in transfected

mammalian cells (16).

We have sought to improve this inducible vector system further by incorporating the advantages of an episomal vector. The presence of the Epstein-Barr virus (EBV) origin for plasmid replication (*oriP*) and the EBV nuclear antigen 1 (EBNA 1) gene on the same DNA molecule allows for extra-chromosomal replication in a variety of established primate cells, but not in rodent cells (20). Such EBV-derived plasmids replicate only once per cell cycle and are not amplified on entry into cells (19); they are maintained at anywhere from a few copies to about one hundred copies per cell under selection in different cell lines, and are lost from the cell population at rates of about 5% per generation in the absence of selection (19,20).

Episomal vectors such as those derived from the EBV replicon have several advantages (8,11,12,14). First, the absence of integration into host-cell chromosomes obviates potential problems of integration position effects on gene expression and reduces the likelihood of any rearrangement of the transfected DNA (8,11,12,14). Second, the expression levels of transfected genes are consistently higher than expression from non-episomal vectors (11). Third, recovery of the transfected episomal plasmids is simple and efficient using the Hirt's extraction procedure (4,9).

Here, we report the construction of an IPTG-inducible episomal expression vector, pEpiLac, which combines the advantages of both the IPTG-inducible expression system and the EBV-based, extra-chromosomal replication system.

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We also introduced the LAP267 transactivator into the human breast carcinoma cell line MCF7, thus establishing a MCF7-derived cell line in which the pEpiLac vector can efficiently express exogenous genes in an inducible manner.

MATERIALS AND METHODS

Cell Culture

MCF7 cells and MCF7/LAP5 cells were maintained at 37°C and 5% CO₂ in MEM containing nonessential amino acids, plus 1 mM sodium pyruvate, 1 mM glutamine (all from Life Technologies, Gaithersburg, MD, USA), 10% fetal bovine serum (Inter-gen, Purchase, NY, USA) and 10

µg/mL bovine insulin (Life Technologies). Cells were refed every 2–3 days. Where indicated, hygromycin B (Roche Molecular Biochemicals, Indianapolis, IN, USA) or G418 (Life Technologies) was added into the medium to the final concentrations of 75 µg/mL or 600 µg/mL, respectively. No G418 selection pressure is required to maintain MCF7/LAP5 cells.

Transfections

For electroporation, 2×10^7 cells in 0.8 mL PBS, pH 7.35, and 20 µg DNA were incubated at room temperature for 10 min, electrically pulsed in a 0.4 cm cuvette (Bio-Rad Laboratories, Hercules, CA, USA) at 960 µFD and 330 V. After 5 min incubation at room tem-

perature, cells were cultured in fresh medium. For calcium phosphate precipitation, 7.5 µg total DNA and 5×10^5 cells were used for each 60 mm plate. Cells were exposed to DNA-calcium phosphate precipitates for 8 h and glycerol-shocked (15% glycerol in 20 mM HEPES-K⁺, pH 7.2) at room temperature for 1 min. Glycerol was removed by washing cells twice with PBS. Thereafter, cells were cultured in fresh medium, and IPTG was added where indicated. In all transfections, pSG5 (Stratagene, La Jolla, CA, USA) DNA was used as carrier to bring up the total DNA amount. For stable transfections, cells were split [1 to 5] into hygromycin B or G418-containing mediums two days after transfection and further selected for 3 weeks.

Construction of pEpiLac

An IPTG-inducible episomal expression vector, pEpiLac1 (Figure 1B), was constructed from the episomal vector pREP4 (Invitrogen, Carlsbad, CA, USA) and the pLac1 vector (Figure 1A). pLac1 was derived from pX12 (15), with a multiple cloning site (MCS) that was modified by the insertion of a pair of oligonucleotides containing three rare-cutting restriction enzyme recognition sites: *FseI*, *SfiI* and *NotI* (Figure 1C). pLac1 was double-digested with *AflIII* and *SacI* and the cohesive ends were filled-in using Klenow DNA polymerase (Promega, Madison, WI, USA). pREP4 was digested with *SalI* followed by Klenow DNA polymerase treatment. The large fragment from pREP4 contains the machinery for DNA replication as well as EBNA 1 and EBV *oriP*. The small fragment from pLac1 contains the IPTG-inducible MMTV basal promoter, MCS and the SV40 polyadenylation signal. pEpiLac1 resulted from the ligation of these two fragments (Figure 1B). pEpiLac3 was derived from pEpiLac1 by modifying the MCS (Figure 1C). Hygromycin B is required for long-term maintenance of cells that are transfected with this episomal vector because episomal vectors do not integrate into host cell chromosomes; in the absence of selection, episomal vectors are lost from the cell populations at rates of close to 5% per generation (19,20).

Figure 1. Schematic representation of IPTG-inducible expression vectors. The physical maps of the pLac1 non-episomal vector (A) and the pEpiLac episomal vector (B) are shown. Sequence of their MCS are shown in panel C. Abbreviations are: *OriP*, origin for plasmid replication of EB virus; EBNA1, EB virus nuclear antigen 1; Hyg, hygromycin B resistant gene; lac, lactose operator sequences; MMTV-P, mouse mammary tumor virus basal promoter; SV40 polyA, SV40 virus polyadenylation signal; M5 and P3, PCR primers used to amplify inserts in the MCS; T7 and SP6, promoters that transcribe DNA sequences in sense orientation and antisense orientation, respectively. *FseI*, *SfiI* and *NotI* are rare-cutting restriction enzyme sites.

Extraction of Episomal DNAs

Episomal DNAs were isolated from MCF7/LAP5 cells using Hirt's extraction method (4,5) with some modifications. Briefly, $1-2 \times 10^6$ cells were harvested in TEN buffer (40 mM Tris-HCl, pH 7.5, 1 mM EDTA and 150 mM NaCl.) washed once with cold PBS and then resuspended in 0.36 mL ice-cold TE buffer (10 mM Tris-HCl, pH 7.2, and 10 mM EDTA). SDS was added to the cell suspension for a final concentration of 1% and incubated at room temperature for 10–20 min after gentle mixing. One-fifth volume of 5 M NaCl was then added, gently mixed and incubated at 4°C overnight. Samples were centrifuged at $15\,000 \times g$ for 30 min at 4°C. The supernatant was collected and extracted once each with phenol, phenol:chloroform (1:1), and chloroform:iso-

amyl alcohol (24:1). Episomal DNA was precipitated with 0.3 M sodium acetate and 2–3 volume of absolute alcohol and dissolved in distilled water.

Protein Assays

For Western blot analysis, cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% Nonidet™ P-40 (Sigma, St Louis, MO, USA), 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1% aprotinin and 10 µg/mL leupeptin) on ice for 10 min, centrifuged at $15\,000 \times g$ for 15 min at 4°C. Protein were quantified using Dc protein assay reagents (Bio-Rad Laboratories), separated in 10% SDS-PAGE and electroblotted. Proteins were detected by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using

Figure 2. IPTG-induced expression of the luciferase reporter gene in MCF7/LAP5 cells. (A) MCF7/LAP5 cells were transiently cotransfected with pPGKβGal (1 µg) together with either pLac1Luc (2 µg) or pEpiLac1Luc (3 µg, about 25% less in molar amount than 2 µg pLac1Luc) using the calcium phosphate precipitation method. Luciferase activities from cell extracts were measured 32 h after IPTG stimulation and normalized with β-gal activities. The normalized luciferase activities from IPTG-treated cells were compared to those from the corresponding untreated cells, which were considered as 1 (indicated by *). (B) A stable population of MCF7/LAP5 cells transfected with pEpiLac1Luc was plated at 1×10^5 cells per 60 mm plate. One group of cells was continuously maintained in medium containing 1 mM IPTG for indicated times. Another group of cells was incubated in medium containing IPTG for 24 h and washed twice with PBS and cultured in fresh medium without IPTG. At each time point, luciferase activities were measured and normalized with protein concentrations in whole cell extracts. Data were shown as representatives of three independent experiments.

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affinity-purified polyclonal antibodies, α -Nur77 (7), α -Cyr61 (13) and p27(C-19) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Both luciferase and β -galactosidase (β -gal) activities were measured using reagents from Promega (Madison, WI, USA) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

IPTG-Inducible Gene Expression in Human Breast Cancer Cells

We have constructed the episomal vector pEpiLac (Figure 1B; see Materials and Methods), that combines features of the IPTG-inducible expression system and the episomal vector system. Inducible expression of exogenous genes cloned in this vector requires a cellular background that supplies the IPTG-dependent transactivator, LAP-

267 (3). We chose to establish an inducible system in the context of the human breast cancer cell line MCF7 because it is one of the most extensively studied cell lines in cancer biology. MCF7 cells were transfected with pX6LAP267 and pWLneo (5 μ g and 0.5 μ g per 100 mm plate, respectively). pX6LAP267 is an autoregulatory vector that expresses LAP267 under an IPTG-inducible promoter, and thus a large amount of LAP267 is produced in the presence of IPTG (Pestov and Lau, unpublished data). pWLneo expresses the neomycin-resistant gene in a constitutive manner (Stratagene). After transfection, cells were selected against G418, and 15 independent, G418-resistant clonal cell lines were isolated.

The efficacy of these clonal cell lines (MCF7-LAP267 cells) to support IPTG-inducible gene expression was evaluated in transient co-transfection assays. These MCF7-LAP267 cells were co-transfected with either of two reporter constructs: pEpiLac1Luc, which expresses the luciferase gene under the IPTG-inducible promoter in the episomal pEpiLac1 vector (Figure 1B); or pLac1Luc, which expresses the luciferase gene under the corresponding promoter in the pLac1 plasmid vector (Figure 1A). These vectors were co-transfected with pPGK β Gal, the β -gal expression vector, which serves as an internal control for transfection efficiencies (1). One clonal line, MCF7/LAP5, showed a high level of IPTG-inducible luciferase activity and was further characterized (Figure 2A).

By contrast, the parental MCF7 cells showed nearly undetectable levels of luciferase activity with or without IPTG treatment (data not shown). Where luciferase activity was induced in pLac1Luc up to about 50-fold under the optimal IPTG concentration, the expression of luciferase was inducible up to 300-fold under similar conditions when expressed using the episomal pEpiLac1Luc (Figure 2A). Thus, the episomal vector pEpiLac1 both maintains a low level of basal expression under uninduced conditions and allows a higher level of IPTG-inducible expression when compared to the non-episomal vector pLac1. We have also established the episomal inducible system in the human glioblastoma cell line U-373

and the human osteogenic sarcoma cell line U2OS, and similar results were obtained (unpublished data).

On-Off Regulation of Gene Expression

Because expression of exogenous genes in the pEpiLac vector can be turned on efficiently by IPTG induction, we examined whether this expression can be turned off following the removal of the inducer. We generated a population of MCF7/LAP5 cells stably transfected with pEpiLac1Luc and treated these cells with 1 mM IPTG to induce the expression of luciferase (Figure 2B). After 24 h of treatment, the cells were either allowed to continue incubation in the presence of IPTG or washed twice with PBS and cultured in fresh medium without IPTG. After IPTG removal, the luciferase activities remained high for about 6 h and then quickly dropped to near basal levels 24 h thereafter (Figure 2B). However, without removal of IPTG, the luciferase activities continued to accumulate until a plateau was reached (Figure 2B). Thus, expression of the luciferase gene from pEpiLac1 was dependent on the presence of IPTG, and this induction was reversible.

IPTG-Regulated Expression of Exogenous Genes in Stable Transfectants

Exogenous luciferase reporter gene was efficiently expressed in both transient and stable transfection assays in MCF7/LAP5 cells (Figure 2). Next, we generated populations of MCF7/LAP5 cells stably transfected with pEpiLac vectors expressing either the p27^{KIP1} CDK inhibitor (18), the orphan nuclear receptor Nur77 (6) or the angiogenic inducer Cyr61 (2). Cells that survived hygromycin B selection was treated with 1 mM IPTG for various durations. The expression of exogenous gene products was detected in Western blot analysis using affinity-purified polyclonal antibodies (Figure 3). Exogenous proteins was detectable as early as 6 h after IPTG treatment (Figure 3). Overexpression of p27^{KIP1} dramatically inhibited cell proliferation rate; cell growth was restored after the removal

Figure 3. IPTG-induced expression of various exogenous genes in stable transfection assays. MCF7/LAP5 cells were transfected with 20 μ g pEpiLac1p27, pEpiLac3Nur77 or pEpiLac3Cyr61 by electroporation. Cells surviving hygromycin B selection were plated in 100 mm dishes (1×10^6 cells each) and stimulated with 1 mM IPTG for indicated times or left untreated (UT). Cells were harvested in TEN buffer, washed twice with cold PBS and lysed in 50 μ L RIPA buffer. Ten microliters of cell lysates were resolved on 10% SDS-PAGE. The expression of exogenous gene products was detected in Western blot analysis using affinity-purified polyclonal antibodies. Exogenous p27^{KIP1} (mouse origin) has a higher electrophoretic mobility than the endogenous human p27^{KIP1}, which served as internal control.

of IPTG inducer (unpublished data). This is consistent with the growth inhibitory effect of the p27^{KIP1} (18). Without IPTG stimulation, exogenous p27^{KIP1} expression was undetectable (Figure 3), which indicates there was no detectable leakage in this system. Exogenous Cyr61 or Nur77 protein levels in cells not treated with IPTG were similar to the basal level of endogenous proteins (data not shown). The low or undetectable level of leakage in this system allows the regulated expression of growth inhibitory genes such as p27^{KIP1} and suggests that this system may be useful for cytotoxic genes.

As is the case with other episomal vectors, the pEpiLac DNA can be maintained extra-chromosomally. When cells transfected with the p27^{KIP1} construct were maintained in media containing hygromycin B for 10 months, episomal DNAs could still be easily harvested using Hirt's extraction (9). After amplification in bacterial cells, the integrity of these episomal DNAs was confirmed by restriction enzyme digestion and PCR analysis (data not shown). Therefore, this system can be applied in genetic selection procedures that require regulated on-off gene expression and recovery of transfected sequences, such as selections using genetic suppressor elements or selectable expression of transient growth arrest phenotype (10,17).

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