Site specific integration of FLP recombinase in BHK-21 cell line

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Received 16 September 2002; revised 1 March 2003

A binary system for gene activation and site specific integration based on conditional recombination of transfected sequences mediated by FLP recombinase from yeast was implemented in mammalian cells. In several cell lines, FLP rapidly and precisely recombined copies of its specific target sequences to activate an otherwise silent beta-galactosidase reporter gene. Clones of marked cells were generated by excisional recombination within a chromosomally integrated copy of the silent reporters. These clones exhibited intense blue colour with X-Gal staining solution.

Keywords: BHK-21 cell line, FLP-recombinase, Recombinase, Saccharomyces cerevisiae

In near future, stable high producer cell lines will be required for insertion of foreign of therapeutic importance such as, erythropoietin by carrying out a single transfection step. Hence the aim of the present study is to develop a stable high producer BHK-21 cell line.

A conditional recombination system based on the site specific recombinase, termed FLP from Saccharomyces cerevisiae has been studied. In these system, gene activation requires prior FLP mediated excisional recombination and expression, therefore falls under the binary control of transgene’s own cis acting sequences and those that direct FLP expression. Reversal of this experimental conditions, provides a means for introducing DNA into specific sites in mammalian chromosomes.

A co-transfection assay is used to characterize FLP mediated recombination of extra chromosomal DNA in a variety of cell lines. Cells are transfected with an expression construct and a ‘reporter’ plasmid that is a substrate for recombinases. The activity of expression construct is assayed either by freeze thaw method or cell lysis method to measure beta-galactosidase activity generated by precisely recombined reporter molecule.

The pNeoβGal reporter plasmid used in these assay was derived from pFRTβGal. pFRTβGal contains the bacterial beta-galactosidase coding sequence, which has been modified by insertion of FLP recombination target site (FRT) immediately 3' to transcriptional start. The FRT site consists of 3 sets of 13 base pair (bp) repeats, 8 bp and 1bp spacer. Inverted repeat pairs of #1 and #2 are essential. The spacer (8 bp) is the target for staggered endonucleotides. The spacer length is important, though the addition of 10bp is tolerated. The sequence of the spacer is not important as long as both sites are the same. The complete FRT sequences site is shown below.

The arrows represent the FLP (13bp) binding elements, and the box spacer (8bp). The two cleavages occur at the border of the binding element and spacer DNA, and are represented by small black arrowheads.

β galactosidase is a useful marker enzyme for many standard molecular biology procedures. The enzyme is both easy to detect and limited in its endogenous expression in mammalian cells, making it ideal for monitoring the success of transfection experiments. The assay of β galactosidase enzyme offers a direct and easy procedure for measuring β galactosidase enzyme activity in cells transfected with galactosidase control vector. Cell extracts are incubated with buffer and substrate O-nitrophenyl β D-galactoside (ONPG). The substrate diffuses into the cell and produces yellow colour. The optical density is measured spectrophotometrically.

X-Gal (5 bromo 4 chloro 3 indolyl-β-D galactoside), an indole derivative, has been successfully employed to stain cell culture and tissue culture preparations. It...
Materials and Methods

Baby hamster kidney (BHK) cells from ATCC, pNeoβGal, POG44 plasmid vectors, Eagles minimal essential medium (EMEM), new born calf serum (NBCS), calcium chloride, neomycin, X-Gal buffer solution, ONPG, were obtained from Sigma Aldrich (USA), HEPES Buffer from Hi Media (Mumbai), phosphate buffer saline from Genei (Bangalore).

The secondary culture of BHK cells was grown in CO₂ incubator (5%) at 35°C to confluence (3 days) in a 25cm² tissue culture flask containing 5ml complete medium (EMEM +10% NBCS). The resulting monolayer was washed with PBS and 1ml of 0.25% trypsin was added to the flask. The flask was left at room temperature for 30 sec. Trypsin (80%) was removed and the bottle was tapped gently to ensure that all the cells detached completely. Complete medium (3ml) was added to these detached cells immediately. Cells were dispersed gently with the help of pipeter. Cell suspension (1ml) was transferred into a culture flask (25cm²) and volume was made up to 5ml by adding 4ml of complete medium.17 18

Cell count was done by trypan blue exclusion method.18 A mixture containing (10 µg/ml of DNA, 50µl of 2M CaCl₂) was added drop wise with a Pasteur pipette to 500µl of 2X HeBS contained in sterile conical tube (15 ml). The conical tube was vortexed immediately for 5 sec and the precipitate was allowed to settle for 20 min at room temperature. The precipitate obtained was distributed (1ml/plate) evenly on to petri plates (60 mm) seeded with 2×10⁵ cells and the plates were incubated for 6 hr in CO₂ incubator (3%) at 35°C. After incubation, the medium was decanted and DMSO shock (3.5 ml) was given to cells for about 3 min to increase the transfection efficiency. After removal of DMSO, cells were fed with complete medium (EMEM +10% NBCS) and incubated for 3 days.18 22 After 3 days, complete medium was replaced by selective medium [EMEM + NBCS (10%) + neomycin (225µg/ml)]. Cells were incubated for 11 days with changes of medium at 3 day intervals. After day 11, the antibiotic concentration in medium was increased to 250µg/ml.

On day 14, the transfected colonies were identified morphologically and marked. Five transfected colonies were chosen at a time, trypsinized and transferred into a 96 well plate. PBS was added to other colonies to prevent drying. This procedure was repeated for all transfected colonies.

Recombinase expression plasmid (POG44) was transfected with BHK/pNeoβGal clones by calcium phosphate transfection for FLP recombinase. The plate was incubated for 48 hr in CO₂ (5%) incubator at 37°C.

Cells were washed twice with PBS and covered with fixing solution [glutaraldehyde (2%) in PBS] for 20 min, washed with PBS, covered with X-Gal staining solution [potassium ferrocyanide (4mM), potassium ferrocyanide (4 mM) and MgCl₂ (2mM)] and incubated for overnight at 37°C. After incubation period the transfected clones exhibited intensive blue colour. β-galactosidase activity was assayed by cell lysis method10 12

Two sets of 2×10³ cells/well of each clone were seeded. One set was treated as a test and other as a control. After overnight incubation each test set was transfected with POG44 vector. The total medium was discarded after 72 hr of transfection and cells were washed with PBS. Twenty µl of 1xCLR [Tris base (250mM), glycerol (50%), Triton X-100 (5%), 1M of dithiothreitol (DTT), 20 mM of 2-diamino cyclohexane tetraammonium chloride (EDTA) and 30 µl of Z buffer [Na₂HPO₄ (0.06M), NaHPO₄ (0.04M), KCl (1M), MgSO₄ (1M), beta mercapto ethanol (BME)] pH (7.0) was added and centrifuged at 3000g at 4°C for 10 min. Supernatant was transferred into a plate having 96 wells and 100µl of ONPG substrate was added. The plate was incubated for 45 min at 37°C. Reaction was terminated by adding 1 M Na₂CO₃(50µl) and readings were taken at 405 nm.

Results and Discussion

Exponentially growing cells (2×10⁵) were seeded in petri plates (60 mm). For transfection with CaPO₄, concentration of plasmid (pNeoβGal) used was 10 µg/ml. Total of seventy eight transfected colonies were obtained, of which 10 were stably transfected neomycin resistant clones (Table 1; Fig. 1). These 10 clones were propagated individually in a 96 well plate. After confluence cells were trypsinized and transferred into 12 well plate. Actively growing cells (2×10³) of each clone were taken in fresh 96 well plate in duplicate. For co-transfection, the concentration of plasmid vector (POG44) used was 10 µg/ml. The stable transfected clones exhibited intense blue colour with X-Gal staining solution (Fig. 2). Of the 10
clones screened, clone 3 exhibited highest β-galactosidase by cell lysis method. This clone has been preserved by cryopreservation technique in liquid nitrogen for further work. The results suggested that FLP could be used to mosaically activate or inactivate transgenes for analysis of vertebrate development, and to efficiently integrate transfected DNA at predetermined chromosomal locations. This stable high producer cell line can be used for inserion of foreign gene of therapeutic importance by carrying out a single transfection step in future.

Acknowledgement

One of the authors (GJP) gratefully acknowledges the Department of Pharmaceutical Sciences, Andhra University, Visakhapatnam, India for providing the infrastructure for this study, and we thank Dr Nasir S Ahmad and Rajesh Guptha Sun Pharma Advanced Research Center, Vadodara, for technical assistance.

References

11 Elaine Schenborn & Virginia Griffon, Luciferase assay for beta Gal, (Promega Corporation, USA).