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Research paper

EXPRESSION OF STREPTOKINASE GENE CLONED IN MAMMALIAN EXPRESSION VECTORS IN BHK21 CELLS

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ABSTRACT: The streptokinase gene cloned in pTarget and replicase based pSinCMV vector was transfected in BHK21 cells. The expression was detected using indirect fluorescent antibody, immunoperoxidase and SDS-PAGE/western blot techniques. High level of gene expression was observed in case of both recombinant plasmids. It shows the functionality of both the recombinant plasmids which could be further evaluated in field trials.

KEYWORDS: Streptokinase, pSinCMV vector, pTarget vector, expression, BHK21 cells

INTRODUCTION

Malke et al (2000) demonstrated several molecular genetic approaches to study the expression of the streptokinase gene. Southern hybridization analysis demonstrated homology of synteny of SKa, SKc and SKg in the genomes of the human subgroups A, group C and group G

streptococci. The S1 nuclease mapping, use of transcriptional fusions to beta-galactosidase and luciferase reporter genes, in conjunction with site-directed mutagenesis, led to the localization of the core promoter region of SKc and the identification of a cis-active upstream region required for full promoter activity.

Due to its fibrinolytic potential, streptokinase is currently used as a thrombolytic therapy drug. The streptokinase protein is being used widely produced in yeast expression vector. However, with advances in recombinant DNA technology, other vectors can be used and analysed for their use.

Wolf et al (1990) demonstrated that direct intramuscular injection of plasmid DNA resulted in expression of the cloned DNA in protein. Hence, this mechanism can be utilized to clone the streptokinase gene in a mammalian expression vector which will produce streptokinase protein which in turn will induce thrombolysis. This procedure will eliminate the adverse reactions sometimes observed on direct injection of streptokinase protein. The cloning of sk gene in replicase based vector will minimize the requirement of DNA to be used and thus will reduce the cost of the product. In addition, cultivation of recombinant cultures containing SK gene on large

scale in fermenter and subsequent purification of plasmid DNA becomes much more economic and efficient as compared to protein purification (Rai et al, 2009).

MATERIALS AND METHODS

Recombinant plasmid

The pTarget.sk10 and pSinCMV.sk11 recombinant plasmids containing human streptokinase gene (Soni et al 2010, 2011) were used.

Cell culture

Baby Hamster Kidney (BHK-21) cell line was obtained from National Centre for Cell Science (NCCS), Pune. This cell line was used in the study for in vitro expression, transfection and analysis of recombinant plasmid and was maintained in DMEM (Gibco, NY) supplemented with 50µg/ml gentamicin (Amresco, USA), 25mM HEPES and 10% fetal bovine serum (FBS). Horseradish peroxidase (HRP) labelled and FITC labelled conjugates were procured from Bangalore Genei, Bangalore.

Isolation of plasmid DNA for transfection

Plasmid DNA isolation was done with Wizard plus SV minprep DNA purification system (Promega, USA). Briefly, a single colony was picked from freshly streaked plate of recombinant plasmid culture and inoculated in 5ml LB medium containing ampicillin and kept for incubation for 8 hour at 37°C in orbital shaking incubator at 200rpm. The starter culture was diluted 1/500 into 3ml selective medium and allowed to grow for 12 to 16 hour at 37°C in orbital shaking incubator at 200rpm. The bacterial cells were harvested at 6000g for 15 min at 4°C. The cells were resuspended in 250µl cell resuspension solution. The cells were lysed by adding 250µl cell lysis solution. Then 10µl alkaline protease solution was added and incubated for 5 min at room temperature. After incubation 350µl neutralization solution was added and then centrifuged at 12000g for 10 min at room temperature. The clear lysate was collected in a spin column fitted with a collection tube and centrifuged at top speed (12000rpm) for 1 min at room

temperature. The flow through was discarded and column was re-inserted into collection tube. 750µl wash solution (ethanol added) was added to the column and centrifugation was done at top speed for 1min. The flow through was discarded and column was reinserted into collection tube. This washing step was repeated once with 250µl wash solution. After washing, spin column was transferred to a sterile 1.5ml microcentrifuge tube. 100µl of nuclease free water added to the spin column and centrifugation was done at top speed for 1 min at room temperature. The column was discarded and DNA was stored at -20°C.

The plasmid DNA was quantified as described earlier.

Hyperimmune serum

Hyperimmune serum against streptokinase was raised in mice using recombinant plasmid. The recombinant plasmid (50µl/mouse) was administered four times at intervals of one week to five mice intramuscularly in the thigh region of hind leg. The blood was collected 7 days later from retro-

orbital plexus of eye of the mouse and serum was collected (fig 8, 9).

Transfection of BHK-21 cell

Cell line was maintained as per the guideline provided along with cells, the cells were maintained in DMEM supplemented with 10% calf serum in 25cm² flasks. The ultrapure plasmid DNA was transfected to BHK-21 cells using lipofectamine-2000 transfection reaction (Invitrogen, USA) according to manufacturers instructions. Briefly 1µl DNA in nuclease free water was added to 3µl of lipofectamine transfection reagent and incubated at room temperature for 20min. During incubation BHK-21 cells were trypsinised using trypsin versene and then 4ml of DMEM containing 10%FCS and gentamycin (50µl/ml) was added to make cell suspension of 1x10⁵ cells/ml. The 100µl cell suspension was loaded into the wells of 96 wells plate containing equally distributed transfection mixture. In three wells, only 100µl cell suspension was added to be used as cell control.

The plate was incubated in airtight dessicator at 37°C for 72 hour at 5% CO₂ tension and humidity.

Indirect fluorescent antibody test (IFAT)

72 hour after transfection, medium from the wells was poured off, cells were washed twice with PBS and fixed with 4% paraformaldehyde. Mouse primary anti-SK hyperimmune serum (1:50 dilution) was added in duplicate wells of each recombinant plasmid, vector alone and healthy groups. It was incubated at 37°C for two hours and then wells were washed with IX PBS and incubated with 1:40 dilution of FITC conjugated goat anti-mouse secondary antibody (Bangalore Genei) for two hour at 37°C, washed with PBS, mounted with 50% glycerol in PBS and examined under fluorescent inverted microscope (Nikon) and photographed.

Immunoperoxidase test (IPT)

The initial steps up to addition of mouse primary antibody were same as describe above. In secondary antibody, HRP conjugated rabbit anti-mouse antibody was added to wells and incubated

for 1 hour at 37°C. The cells were again washed with PBS thrice and incubated with substrate (3,3-diaminobenzidine, DAB 1mg/ml in PBS with 1µl/ml H₂O₂) for 5 min. After the development of colour, cells were washed with PBS, dried in air and observed under inverted microscope and photographed.

SDS-PAGE/ Western blot analysis

BHK-21 cells were transfected in 25 cm² flask with recombinant plasmids (5µg) and vector (5µg) separately. After 72 hours of transfection, cells were processed following the method of Sambrook and Russel, (2001). Briefly, cells were trypsinized with 0.5ml of trypsin versene and pelleted in 1ml PBS by centrifugation at 8000rpm for 4min. The cells were resuspended in extraction buffer, freeze thawed and supernatant was aliquoted in 40µl volume in tubes. Laemmli buffer was added to each aliquot to a final concentration of 1X, boiled for 5min and loaded on 10% polyacrylamide gel in parallel with protein molecular weight marker in SDS-PAGE vertical slab gel apparatus. After

completion of electrophoresis, MW marker and one lane was cut out and stained with Coomassie brilliant blue while the protein band from unstained gel were blotted onto nitrocellulose membrane using semi-dried system apparatus (Atto) at 2mA/cm² for 90 min. Further processing was done in SNAP id protein detection system (Millipore) following the manufacturers protocol as described below.

Blot assembly

Opened the blot holder lid taking care not to damage the inner white face. Wet the inner white face of the blot holder with milli-Q water, until it turns gray. Removed excess liquid using the blot roller. This will prevent movement of the blot during assembly. Placed the pre-wet blot membrane in the center of the blot holder, protein side down. Rolled gently with blot roller to remove any air bubbles trapped between the blot holder and the blot. Placed the spacer (wetting not necessary) on top of the blot, making sure it completely covers all edges. Rolled blot again to ensure complete contact of blot spacer with blot membrane and closed the

blot holder lid. Squeezed firmly at the base of the tab area to secure lid. Opened lid of system by squeezing latch between thumb and forefinger and lifting upwards, placed blot holder in system chamber with well side up aligning the blot holder tabs with notches of chamber.

Immunodetection protocol

Added 10ml blocking solution (0.5% skimmed milk in PBS-T) per well being used. After the well have emptied completely (10-20 sec) turned vacuum off using the vacuum control knob. Added 1ml mouse anti-SK antibody to each well. Antibody solution must evenly cover entire blot holder surface. Incubated for 10 min at room temperature with vacuum off. Turned the vacuum on and waited 10-20 sec to make sure that antibody solution has been completely emptied from the blot holder. With vacuum running continuously, washed the blot with 10-30ml of wash buffer PBS. Three sequential washes were done. When the blot holder was empty, turned vacuum off. With the vacuum off, applied the secondary antibody HRP conjugated rabbit anti-mouse antibody evenly across the entire

surface of the blot holder. Incubated for 10 min at room temperature with vacuum off. Turned the vacuum on and waited 10-20 sec to make sure that antibody solution has been completely emptied from the blot holder. With vacuum running continuously, washed the blot with 10-30 ml of wash buffer PBS. Three sequential washes were done for optimal performance. Removed blot holder from the system, placed it on the bench with well side down and opened the lid. With forceps removed and discarded the spacer. Remove blot and incubate with substrate solution DAB-H202 (1mg/ml DAB, 24 μ l/ml H202, 20 μ l/ml nickel chloride (8% in PBS)), Bangalore Genei and observed in white transilluminator (Medox) and photographed.

RESULTS AND DISCUSSION

The expression of SK gene was studied using both the recombinant plasmids pTarget.sk10 and pSinCMV.sk11 by transfecting BHK-21 cells and detecting the expressed protein using immunofluorescence, immunoperoxidase, SDS-PAGE and immunoblot techniques. The

pTarget.sk10 transfected BHK-21 cells showed good immunofluorescence (Fig.1) while control cells did not show fluorescence (Fig.2), similarly the pSinCMV.sk11 transfected BHK-21 cells showed intense immunofluorescence (Fig.3) while control cells did not show any fluorescence (Fig.4). The pTarget.sk10 and pSiinCMV.sk11 transfected BHK-21 cells showed intense positive colour reaction on immunoperoxidase test (Fig.5-6) while control cells did not show any colour development (Fig.7). Both the recombinant plasmids showed protein expression in BHK-21 cells when the transfected cells were analysed on SDS-PAGE (Fig.8) and were further confirmed by immunoblot test in which both proteins showed positive colour reaction on immunoperoxidase staining reaction.

Expression studies on SK gene in BHK-21 cell culture using immunofluorescence and immunoperoxidase techniques revealed that high level of gene expression was achieved by both the recombinant plasmids 72 hours after transfection.

The immunoperoxidase test was found to be very useful in analysis of gene expression since it

involves neither any fluorescent dye nor fluorescent microscope since the results are observed in ordinary laboratory inverted microscope which can be photographed as well as the microtitre plates can be stored permanently for future observation, record as well as for teaching purposes.

The expression was also analysed by SDS-PAGE which showed the presence of SK protein band and it was further confirmed by immunoblotting experiment. The analysis of the SK protein using these parameters confirmed that the SK protein will be made in sufficient amount in mammalian system when the recombinant plasmid will be injected and therefore the recombinant plasmid will be of great use for therapeutic purposes. Moreover the pSinCMV.sk11 recombinant plasmid will be of special importance since it will be required in quantities of 1 or 2 μg per person which will make it very economic and will be the product of choice in Indian conditions. Malke et al (2000) demonstrated several molecular genetic approaches to study the expression of the streptokinase gene. Nikandrov et al (2008) studied

the effects of plasminogen and streptokinase on the vital functions of nervous tissue cells in culture. In the protein-deficient media plasminogen stimulated the vital functions of cells and it protected cells of sympathetic ganglia, neocortex and continuous cell lines under damaging actions of H₂O₂ (0.001M), NH₄Cl (0.01M) and cooling. Streptokinase essentially influenced the mode of damaging effect of ATP (0.001M). Even a short-term exposition (20 min) of PC12 cells with both proteins led to sharp alterations in intracellular ATP- or Ca²⁺-activated proteolysis. In some cases plasminogen and streptokinase provided acceleration of cultured tissue maturation, improvement of cell adhesion, high survival rate, the increase in quantity and length of processes and their arborisation. Electronic microscopy established the character of structural rearrangement of nervous tissue cells (neurons, astrocytes, oligodendrocytes), reflecting the protective action of plasminogen and streptokinase. In the presence of plasminogen and especially streptokinase, the total number of

cultured glioma C6 and neuroblastoma IMR-32 cells, the intracellular contents of protein, RNA and DNA increased several-fold. Addition of plasminogen promoted formation of processes by neuroblastoma cells; this suggests initiation of differentiation of cellular elements. In cultures of sensitive and sympathetic ganglia streptokinase increased proliferation of Schwann cells. These proteins did not cause transformation of PC12 enterochromaffine cells to neurons, though plasminogen facilitated it. Plasminogen addition to cell cultures did not increase fibrinolytic activity of the culture medium, and streptokinase did not lose its plasminogen-activating capacity.

In the present study, pSinCMV, a replicase based vector, was used which is superior over other conventional vectors in terms of its lower requirement of DNA (Xiong et al, 1989; Hariharan et al, 1998; Bergland et al, 1998; Leitner et al, 2000) and the power of inducing apoptosis (Leitner et al, 2003) so that transient but robust expression of antigen is achieved in short period of time without taking risk of integration into the host chromosome

(Jolly, 1994; Miller et al, 1993; Samulksi et al, 1989), and transfection of non- dividing cells (Strauss et al, 1994). The pSinCMV is an alpha virus (Sindbis virus) based plasmid vector which serves as negative strand once it enters into the host cell. After entering into the nucleus it is transcribed by host RNA polymerase enzyme from CMV promoter into a full length RNA transcript. This full length transcript then acts as positive sense alpha virus which in turn is translated into protein.

Since the cloned insert is downstream to the subgenomic promoter, it makes large number of target mRNA from the sequence downstream to it. If this recombinant *E. coli* culture is grown in large scale fermenters and then plasmid DNA is prepared, it would cost less and would be commercially viable. Rai et al. (2009) demonstrated that recombinant *E. coli* culture could be grown in 10 L volume in fermenter and then plasmid DNA could be isolated and purified using indigenously optimized silica gel DNA purification technology.

The plasmid DNA thus prepared was found to be sterile, safe and potent and could be stored at room temperature, obviating the need of cold chain. Such a scenario is highly desirable in Indian conditions since we do not have a cold chain and any product not requiring a cold chain is valuable and would be highly economic.

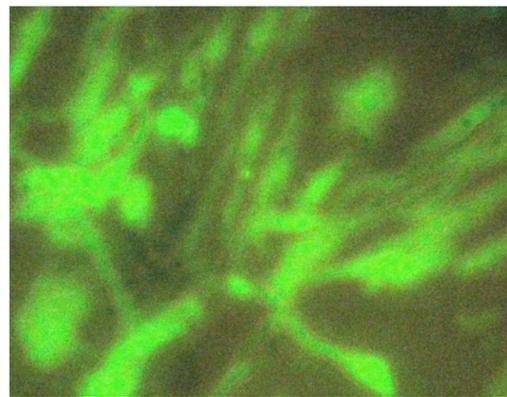


Fig.1. Expression of pTarget.sk 10 in BHK21 cell culture using FAT showing positive gene expression.

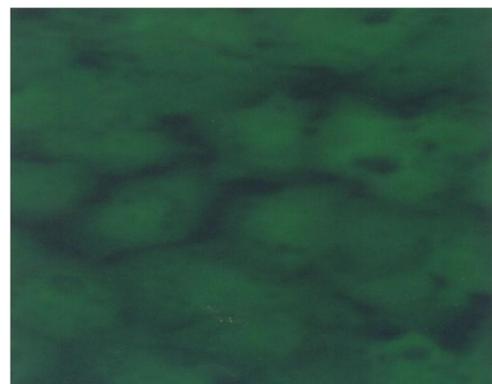


Fig.2. Mock transfected BHK21 cell culture showing no fluorescence

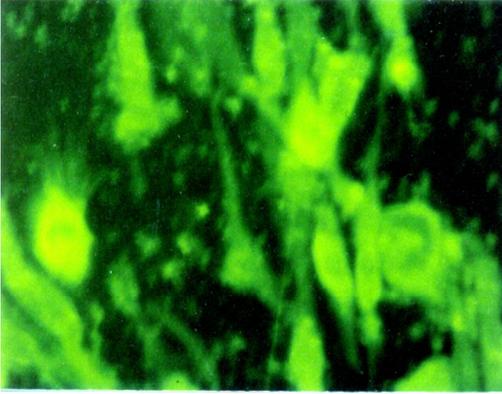


Fig. 3. Expression of pSinCMV.sk 11 in BHK21 Cells by FAT.

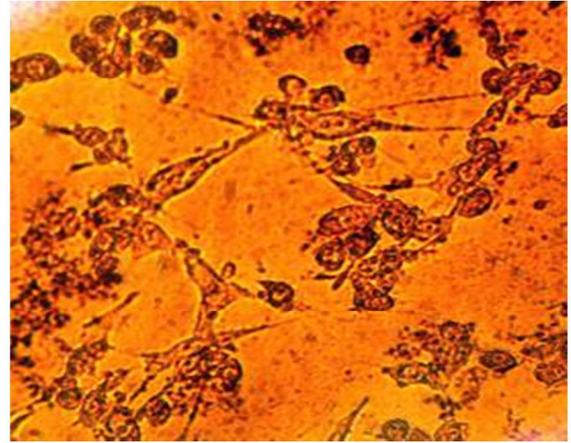


Fig.6. Expression of pSinCMV.sk 11 in BHK21 cell culture using IPT.



Fig.4. Mock transfected BHK21 cells showing no fluorescence.

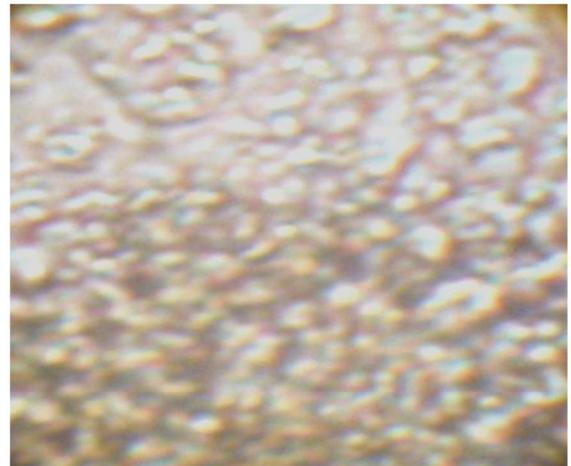


Fig.7. Control BHK21 cell culture.

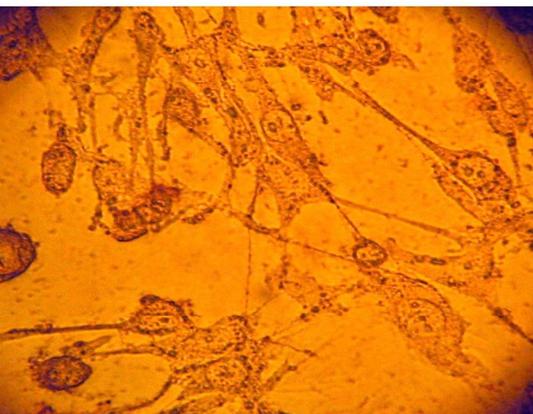


Fig.5. Expression of pTargetTsk10 in BHK21 cell culture using IPT.

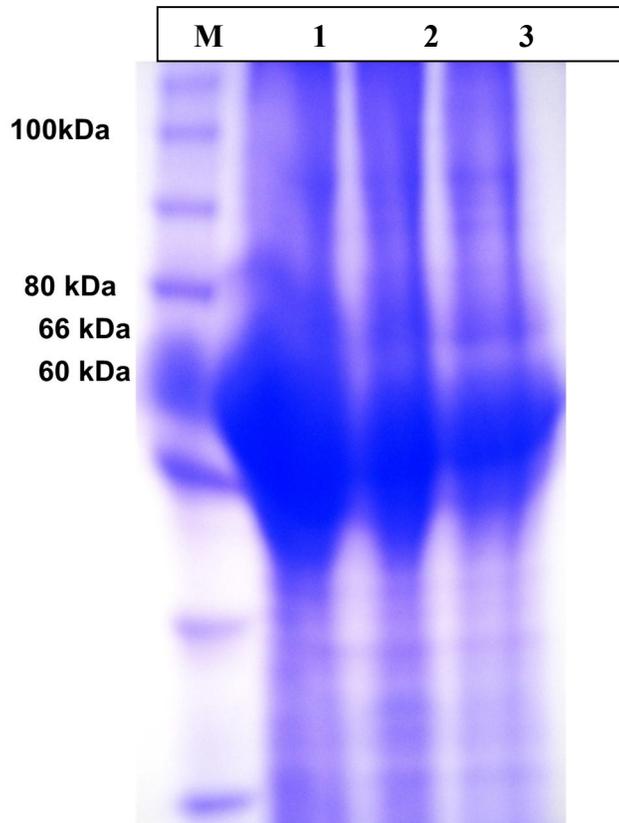


Fig.8. Detection of sk protein expression by SDS-PAGE(arrow), Lanes M: Protein MW marker; 1: Control non-transfected cells; 2: Cells transfected with pTarget.sk10; 3: Cells transfected with pSinCMV.sk11

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