Biotechnology International 4(2): 31-35, June 2011
ISSN 0974-1453
Published by Biotechnology Society Online www.bti.org.in

Cloning of human erythropoietin gene in pSinCMV vector

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Summary: The erythropoietin (epo.hu) gene already cloned in pTarget vector was released by digesting with *EcoRI* and cloned in *StuI* site of pSinCMV vector. The clone containing gene in right orientation was confirmed using RE digestion, PCR and sequencing and designated as pSinCMV.epo.hu.

Keywords: Erythropoietin gene, pSinCMV vector, replicase vector, gene cloning

Introduction

Erythropoietin is a glycoprotein hormone that controls erythropoiesis or red blood cell production. It is a cytokine for erythrocyte (red blood cell) precursors in the bone marrow also called hematopoietin or hemopoietin, it is produced by the capillary endothelial cells in the kidney and liver, it is the hormone that regulates red blood cell production. It also has other known biological functions. For example, erythropoietin plays an important role in the brain's response to neuronal injury. Erythropoietin is also involved in the wound healing process (Lin et. al.,1985). Erythropoietin expression increases in five-sixths nephrectomized rats, after muscle-targeted gene transfer by in vivo electroporation, using plasmid DNA expressing rat epo (pCAGGS-epo) (Ataka et.al.,2003). Myelodysplastic syndrome (MDS) may be induced by certain mutagenic environmental or chemotherapeutic toxins; however, the role of susceptibility genes remains unclear. The G/G genotype of the single-nucleotide polymorphism (SNP) rs1617640 in the erythropoietin (epo) promoter has been shown to be associated with decreased epo expression (Ma et. al., 2010). Keeping the above facts in view, the present work was undertaken to clone the human erythropoietin gene in replicase based eukaryotic pSinCMV vector.

Materials and Methods

Vectors: pTarget.epo.hu. recombinant plasmidand pSinCMV vector were available in

Biotechnology Laboratory of IBIT, Bareilly.

***E.coli* culture:** Escherichia coli DH5α host strain for transformation of recombinant plasmids was available in the lab.

Isolation of plasmid DNA: Revival of the *E.coli* culture containing pTarget vector with erythropoietin gene was done in LB broth and plasmid DNA was isolated following TELT method (He et. al., 1990). Grown 1.5 ml *E.coli* culture with plasmid in LB medium containing 100µg/ml ampicillin for 16 h. Pelleted cells for 30 sec in microcentrifuge and resuspended in 100 µl TELT solution and added an equal volume of 1:1phenol/chloroform. Vortexed vigorously for 15 sec and spinned 1 min in microcentrifuge at 22^0C . Collected the upper phase of nucleic acid and mixed with 2 vol of 100% ethanol. After 2 min, spinned 10 min in microcentrifuge at 4^0C. Washed pellet with 1 ml of 70% ethanol, allowed to dry and resuspended in 30 µl TE buffer. Plasmid DNA was checked on 1% agarose gel electrophoresis.

RE digestion and DNA extraction from agarose gel: RE digestion of pTarget.epo.hu with *EcoRI* was done. The gel extraction of DNA fragment was done using MinElute gel extraction kit (Qiagen, Germany) following manufacturers instruction.

Blunting of *EcoRI* generated human erythropoietin gene staggered ends: For blunting of staggered ends generated by *EcoRI* enzyme, T4 DNA polymerase was used. Reaction mixture consisted of epo gene insert 20 µl, T4 DNA polymerase buffer 10X 5 µl, T4 DNA polymerase 2 µl, dNTP mix. (10mM each) 1 µl, nuclease free water 22 µl, making total volume 50 µl. The reaction mixture was incubated at 37˚C for 10 min.

Purification of blunted insert: The blunted epo gene insert was purified using phenol chloroform, following the protocol of Sambrook and Russell (2001).The presence of purified blunted DNA was checked by running 1 µl of DNA on 1% agarose gel.

Preparation of pSinCMV vector: The pSinCMV vector DNA was digested with *StuI to* create blunt end using 50 µl reaction mixture. The linearised plasmid was checked on 1% agarose gel electrophoresis. It was than dephosphorylated using Calf Intestinal Alkaline Phosphatase (CIAP) to prevent self-ligation. Purification of dephosphorylated linearized vector was done using phenol chloroform as per method of Sambrook and Russell (2001).

Blunt end ligation of pSinCMV vector and epo gene: The reaction mixture consisted of 30% PEG 8000 for blunt end ligation. The reaction mix consisted of: T4 DNA Ligase (Fermentas) 2.0 µl, pSinCMV vector 0.5 µl, epo gene 4.0 µl , ligation buffer (10x) 1.0µl, 30% PEG8000 (Amresco) 1.5 µl nuclease free water 1.0 µl, making total volume to10 µl in a microfuge tube. The reaction was incubated overnight at 15˚C.

Transformation of *E.coli* (DH5α) cells with the ligated product: The single step method of competent cell preparation and transformation was used (Chung et. al., 1989).

Screening of recombinant clones: Few colonies were picked from the overnight grown transformants. The individual colonies were inoculated in fresh ampicillin

(50µg /ml) containing LB broth and allowed to grow for 18 to 24 hours. Plasmid was isolated from these colonies by TELT method. Plasmid was checked on 1% agarose gel electrophoresis.

Digestion of plasmid with *kpnI* enzyme to check the presence and right orientation of insert: Isolated plasmid was checked for the presence of insert and its right orientation by digestion with enzyme *kpnI* .The digestion mixture (15 µl) contained: Plasmid 4.0 µl, *kpnI* 1.0µl, buffer (10X) 1.5 µl, nuclease free water 8.5 µl. The reaction mixture was vortexed and spun and kept in incubator at 37˚C overnight. The digested mixture was then electrophoresis in 1.5% agarose. The released fragments after digestion were compared against 1 kb marker.

Colony PCR: The presence of gene insert in right orientation in the recombinant plasmid was confirmed by PCR using gene specific forward primer and BGH as reverse primer. The PCR reaction mixture (50 µl) contained 100 ng of recombinant plasmid, 50 pmol each of gene specific forward primer and BGH as reverse primer and 3 units of Taq DNA polymerase in 1x PCR buffer. The reaction was carried as follows: distilled water 33 µl, rplasmid 5 µl, Forward primer of epo gene 2 µl, BGH Reverse primer 2 µl, dNTPs 2 µl, buffer (10X) 5 µl , Taq DNA polymerase 1 µl. The epo gene was amplified following initial denaturation at 94˚C for 5 min and 30 cycles of denaturation at 94˚C for 30 seconds, annealing at 50˚C for 50 seconds, amplification at 72˚C for 7 min and final amplification at 72˚C for 10 min. After amplification, an aliquot of 10 µl was subjected to 1% agarose gel electrophoresis along with 100 bp DNA molecular weight marker.

Sequencing: The recombinant plasmid selected after the above two methods was sequenced using BGH primer.

Results and Discussion

The epo gene already cloned in pTarget vector was released by digesting with *EcoRI* enzyme and the plasmid DNA was gel eluted and purified. It was successfully cloned in pSinCMV vector using blunt ligation method. Vector sequence along with the cloned insert was analysed in Mapdraw using DNASTAR software to find the restriction enzymes, which could release the product so as to identify the plasmid containing gene in right orientation. The enzyme *kpnI* was chosen which released three fragments viz. 0.152kb, 3.359 kb and 7.856 kb if the gene was in right orientation. The fragment sizes obtained were in agreement with the prediction from Mapdraw analysis, which confirmed that the gene was in right orientation. PCR with epo gene specific forward primer and BGH as reverse primer further confirmed that clone was in right orientation. Sequencing of the rplasmid further confirmed that the gene was in right orientation. This recombinant plasmid was designated as pSinCMV.epo.hu. pSin is derived from an alpha virus (Sindbis virus). The sub genomic promoter of alpha virus is very strong so that it makes large number of target mRNA from the sequence downstream to it. Erythropoietin (epo) genomic gene was also cloned and its expression vector pOP13/epo was constructed (Cui et.al., 1998). In another study, A 600 bp synthetic erythropoietin gene encoding all 166

amino acids of the epo protein and 27 amino acids of the signal peptide had been constructed. The results indicated that the nucleotide sequence of the synthetic epo gene was identical to that of the original (Yi et. al., 1992). In comparative studies of conventional (nonreplicating) plasmid DNA vectors and alpha virus DNA-based replicon vectors the latter generally produces larger quantity of DNA concentrations than does conventional vectors.

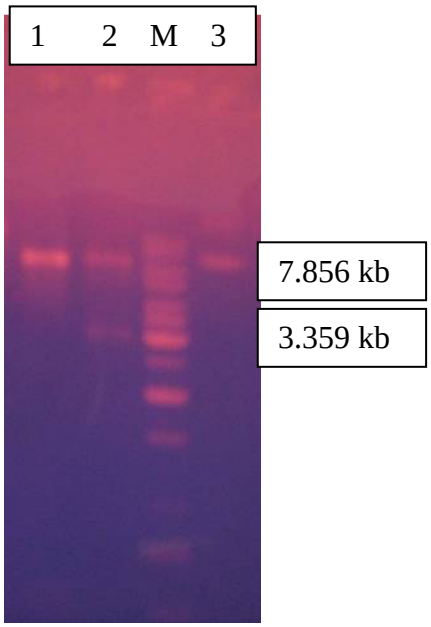

Fig.1. RE digestion of pSinCMV.epo.hu with KpnI . Lane M, 1kb DNA ladder; 1 &3, undigested rplasmid; 2, rplasmid cut with KpnI producing fragments of 3.359, 7.856 kb

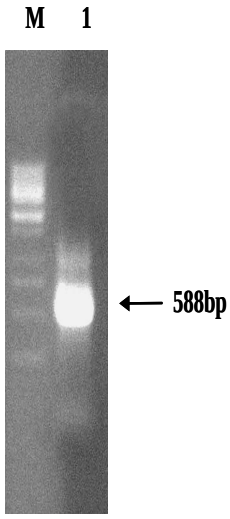

Fig.2. Lanes M: 1 kb ladder; 1: Epo 588 bp PCR product

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