Biotechnology International 4 (1): 16-21, Mar 2011
ISSN 0974-1453
Published by Biotechnology Society Online www.bti.org.in

Cloning of chicken anemia virus VP3 gene in pSinCMV vector

Priyanka Pal *, Kusum Agarwal *, Anant Rai ** and Usha Tiwari *

* School of Biotechnology, Shobhit University, Modipuram, Meerut- 250110
** Institute of Biotechnology and IT, 197, Mudia Ahmadnagar, Pilibhit Road, Bareilly- 243122
*Corresponding author, needpriyanka@gmail.com

Summary: Chicken anemia virus (CAV) is an important pathogen of chicken worldwide, causing severe anemia and immunodeficiency. Its small single-stranded DNA genome (2.3 kb) encodes three proteins: VP1, the only structural protein, VP2, a protein phosphatase, and VP3, also known as apoptin, which induces apoptosis. The VP3 gene of chicken anemia virus, already cloned in pTarget vector was released by digesting with *Eco*RI and cloned in *Stu*I site of pSinCMV vector (blunt end cloning). The clone containing gene in right orientation was confirmed using RE digestion, PCR and sequencing and designated as pSinCMV.cav.vp3.

Keywords: VP3 gene, apoptin, apoptosis, replicase vector, gene cloning.

Introduction

Chicken anemia virus (CAV) belongs to the genus Gyrovirus of the family Circoviridae. It is the causative agent of chicken infectious anemia, primarily an immunosuppressive disease of young chickens, but which also affects all age groups. (Farkas *et al.*,1998). The chicken anemia virus-derived VP3 ("apoptin") protein induces apoptosis in chicken mononuclear cells (Zhuang *et. al.*, 1995). Apoptin, a protein of 121 amino acids, has an intrinsic specificity that allows it to selectively kill tumor cells, irrespective of the p53 or Bcl-2 status of these cells. Hence, it is attractive to explore the use of the apoptin gene for therapeutic applications, viz cancer gene therapy (Pietersen *et. al.*, 1999). New cancer therapies are under development to bypass the resistance to chemo- and radiotherapy of tumors. Apoptin acts independently of p53, is stimulated by Bcl-2 and is insensitive to BCR-ABL, which means that apoptin can induce apoptosis in cases where present (chemo)-therapeutic agents, unfortunately, will fail. The fact that apoptin induces apoptosis in human tumorigenic cells but not in normal diploid cells implies that side effects of apoptin treatment are expected to be minor (Pietersen *et. al.*, 2000). Keeping

the above facts in view, the present work was been undertaken to clone the VP3 gene of chicken anemia virus (CAV) in replicase based eukaryotic pSinCMV vector.

Materials and Methods

Vectors: pTarget.cav.vp3 recombinant plasmid, 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 VP3 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 1:1 phenol/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 4^0 C. Washed pellet with 1ml of 70% ethanol, dried under vacuum, and resuspended in 30 µl TE buffer. Plasmid was checked on 1% agarose gel electrophoresis.

RE digestion and DNA extraction from agarose gel: Then RE digestion of rplasmid pTarget.cav.vp3 with EcoRI was done. The gel extraction of DNA fragment was done using MinElute gel extraction kit (Qiagen, Germany) following manufacturers instruction. Briefly, the agarose gel containing DNA fragment was excised with a scalpel and weighed in a colorless microfuge tube. Three volumes of buffer QGH was added to 1 volume of gel and incubated at 50˚C until gel slice had completely dissolved. One gel volume of isopropanol was added to the melted agarose and mixed by inverting several times. The sample was applied to the min Elute column fitted in a collection tube. The column was centrifuged at 13000 rpm for 1 min and the flowthrough was discarded. 500 µl of QG was applied to the spin column, centrifuged and the flowthrough was discarded again. The DNA bound to the column was washed with 750 µl of buffer PE and the flow through was discarded. The DNA was eluted from the column in 10 µl nuclease free water.

Blunting of *Eco*RI generated VP3 gene staggered ends: For blunting of staggered ends generated by *Eco*RI enzyme, T4 DNA polymerase was used. Reaction mixture consisted of Insert- 20 µl, T4 DNA polymerase buffer 10X- 5 µl, T4 DNA polymerase- 2 µl, dNTP mix. (10mM each)- 1µl, 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 VP3 insert was purified using phenol chloroform precipitation method, following the protocol of Sambrook and Russell (2001). In brief, 50 µl of TE buffer was added to the reaction mixture followed by addition of equal volume of phenol

chloroform isoamyl alcohol (25: 24: 1) i.e.100 µl for above reaction mixture. It was centrifuged at 13000 rpm for 10 min. The aqueous layer was transformed into a different tube. Again 50 µ of TE buffer was added to the tube containing phenol chloroform isoamyl alcohol and centrifuged at 13000 rpm for 10 min. Aqueous layer was transferred to the collection tube. 1/10 volume (5 µl) of 3M sodium acetate was added to the aqueous collection. Then 2.5 times of 100% ethanol was added to it. The tube was kept at –20˚C overnight. It was centrifuged at 13000 rpm for 20 min. Supernatant was carefully discarded and pellet was washed in 1ml of 70% ethanol by centrifuging at 13000 rpm for 10 min. The dried pellet was then dissolved in 12 µl of nuclease free water. The presence of purified blunted DNA was checked by running 1µl of DNA on 1% agarose gel.

Preparation of pSinCMV vector: Growth of rE.coli cells containing pSinCMV vector was done in LB broth, as well on LB agar plates. StuI was used to create blunt end using 50µl reaction mix. The linearised plasmid was checked on 1% agarose gel electrophoresis.

Dephosphorylation of vector DNA: The pSinCMV vector was dephosphorylated using Calf Intestinal Alkaline Phosphatase (CIAP) to prevent self-ligation. Purification of dephosphorylated linearized vector was done as used in purification of blunted insert.

Blunt end ligation of pSinCMV vector and VP3 gene: The reaction mixture consisted of 30% PEG 8000 for blunt end ligation. The reaqction mixconsisted of: T4 Ligase (Fermentas) - 2.0 µl, pSinCMV vector- 0.5 µl, VP3 gene- 4.0 µl, ligationbuffer (10x)- 1.0 µl, 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). A fresh overnight culture of *E.coli* (DH5α) was diluted into prewarmed LB broth and the cells were incubated at 37˚C in shaking incubator. An equal volume of ice-cold 2x TSS was added and the cell suspension was mixed gently. (1X TSS is LB broth with 10% PEG335 or 8000, 5% DMSO, and 20-50mM MgSO4 (or MgCl2) at a final pH of 6.5.) For long-term storage, cells were frozen immediately in a dry ice/ ethanol bath. They were stored at -20˚C. For transformation, a 0.1 ml of aliquot of cells was pipetted into a cold polypropylene tube containing 1 µl of plasmid DNA and the cell/ DNA suspension was mixed gently. The cell/ DNA mixture was incubated for 5-7 minute at 4˚C. A 0.9 ml aliquot of TSS (or LB broth) and 20mM glucose was added and the cells were incubated at 37˚C in shaking incubator. The above media containing transformed cells were plated on LB agar plates containing appropriate antibiotic (ampicillin 100 µg/ ml) and the plates were incubated at 37˚C for 16-24 hours.

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 *Bgl*II enzyme to check the presence of CAV-VP3 insert: Isolated plasmid was checked for the presence of insert and its right orientation by digestion with enzyme *Bgl*II. The digestion mixture contained: Plasmid 4.0 µl**,** BglII 1.0 µl, buffer (10X) 1.5 µl, nuclease free water 8.0 µl. The reaction mixture was vortexed and spun and kept in incubator at 37^0C overnight. The digested mixture was then electrophoresed 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 VP3 2 µl, BGH Reverse primer 2 µl, dNTPs 2 µl, buffer (10X) 5 µl, Taq DNA polymerase 1µl. The VP3 gene was amplified following initial denaturation at 94^0C for 5 min and 30 cycles of denaturation at 94^0C for 30 seconds, annealing at 50^0C for 50 seconds, and amplification at 72^0C for 7 min and final amplification at 72^0C 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 at Chromous Biotech, Bangalore, India.

Results and Discussion

The plates containing recombinant *E.coli* culture contained pTarget.cav.vp3 plasmid, from which few discrete colonies were selected randomly and grown in LB broth containing 100 µg/ml ampicillin. The recombinant plasmid was isolated and was run on agarose gel electrophoresis, which yielded good amount of DNA. The cav.vp3 gene already cloned in pTarget vector was released by digesting with *Eco*RI enzyme and the plasmid DNA was gel eluted and purified. Vector sequence along with the cloned insert was analysed in Mapdraw using DNASTAR software to find the restriction enzymes, which could release the product of desired length. The enzyme *Bgl*II was chosen which released three fragments viz. 5650 bp, 2180 bp and 3321 bp 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. Colony PCR with VP3 gene specific forward primer and BGH as reverse primer further confirmed that clone was in right orientation (Fig. 1). Sequencing of the rplasmid clone further confirmed that the gene was in right orientation. This recombinant plasmid was designated as pSinCMV.cav.vp3.

The VP3 gene was successfully cloned in pSinCMV vector. pSin is derived from an alpha virus (Sindbis virus). The subgenomic promoter of alpha virus is very strong so that it makes large number of target mRNA from the sequence downstream to it. In comparative studies of conventional (nonreplicating) plasmid DNA vectors and alphavirus DNA-based replicon vectors, the latter generally produces larger quantity of DNA concentrations than does conventional vectors.

Future antitumor therapies could use apoptin either as a therapeutic drug or as an early sensor of druggable tumor-specific processes (Backendorf *et. al.*, 2008). For improved treatments to be developed, the ability to target tumor cells selectively is essential, allowing a greater dose of therapeutic agent to be delivered without adversely affecting non-malignant tissue and further studies are required to exploit its therapeutic potential.

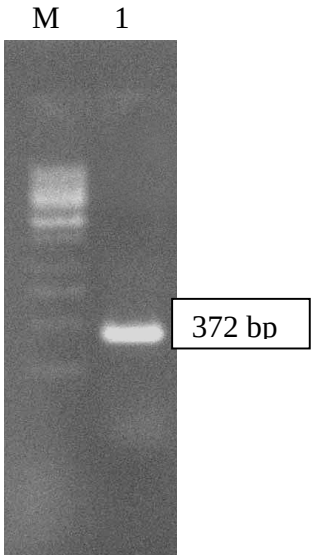

Fig. 1: PCR amplification of VP3 gene.
Lane M: 100 bp DNA ladder; **1:** CAV-VP3 PCR product of 372 bp.

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