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DEVELOPMENT OF DOUBLE GENE MAMMALIAN EXPRESSION VECTOR

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ABSTRACT

Recombinant vectors are valuable tools in the biopharmaceutical industry with a number of novel vectors being developed every day. These Antibodies are often expressed in mammalian cells by co-transfecting light chain and heavy chain containing plasmids. However, co-transfection can lead to variable in the copy number of both heavy chain and light chain, there by affecting the protein productivity. Double gene expression vectors can overcome these problems. In the current design The first transcriptional unit comprises sequences for the CMV promoter, multiple cloning site (MCS) and a Polyadenylation Sequence and the second transcriptional unit comprises sequences for the SV40 promoter, MCS and polyadenylation sequence. The double gene expression vector (pUB-C-S) was constructed by ligating the BglII and BamHI fragment (974bp) form pSI plasmid with BglII digested pCI plasmid. The presence of two independent transcriptional units in the recombinant plasmid was confirmed by colony PCR.

Key Words: Recombinant vectors, biopharmaceutical industry, co-transfection, promoter, multiple cloning site.

INTRODUCTION

Over the past few decades the rapid progress in biotechnology has made it possible to improve the expression of recombinant proteins. However, the current plasmid design still limits the controlled expression of multiple genes.

Several strategies of multiple gene expression were tried in Bacteria, Mammalian and Plant cell (LEE J.H*et al* 2002and Johnston *et al* 2000 and Ruker.p*et a*l 1997 and Susan *et al* 2002). However all these approaches suffer from advantages as well as disadvantages. Off these methods co-transformation with linked transgenes in single vector is a conventional and reliable approach in most of the cases. However, this approach is technically demanding. The lack of unique restriction cloning sites, loss of direct selection, as well as the relatively low efficiency of ligation of insert into larger vector. In order to overcome these problems a novel expression vector was developed with unique restriction sites and two different inducible promoters to control the expression of both the genes.

In the current project I have tried to develop a mammalian double gene expression vector for use in the expression of monoclonal antibodies. The 1st transcriptional unit contains the (a) Cytomegalo virus promoter, a strong constitutive viral promoter widely used in biopharmaceutical production, (b) Multiple Cloning Site (MCS) and (c) Polyadenylation Sequence for transcriptional terminators. The 2nd transcriptional unit consists of (a) SV40 promoter, a constitutive viral

promoter widely used in the expression of selectable markers and recombinant proteins, (b) Multiple cloning site (MCS) with unique restriction sites and (c) poly adenylation sequence for transcriptional terminator. The transcriptional units will help the transcription of both light chain and heavy chain of monoclonal antibody form the same plasmid there by overcoming the problems that were often noticed with the co trnasfection of light chain and heavy chain expression plasmid.

MATERIALS AND METHODS

Isolation of plasmid : Isolation of plasmidDNA from single colony of bacteria was seeded in 10ml of LB media containing 100ug/ml Amp, by centrifugation method using mini prep kit. And elute was transferred in a fresh eppendorf tube and stored at -20°C.

Purification of plasmid : Restriction digestion, PCR samples were routinely purified using UB-Desalting Kit contain desalting buffer and Elutes were collected in a fresh eppendorf tube and stored at -20°C.

Gel extraction : DNA fragments separated on 1-2% agarose gels were purified using UB-Gel extraction kit and elute was transferred into afresh microfuge tube and stored at -20°C

Restriction digestion and Dephosphorylation: conducting restriction digestion by using the given Set up.

Ligation:
50-100ng of DNA was routinely used to ligate with 1-5 U of T4-DNA ligase in 10 µl reaction volume under the reaction conditions specified by the manufacturer. The ratio of vector to insert was maintained between 1:3 and 1:5 for efficient ligation of insert with the vector. The reaction was routinely carried at 4˚C overnight in 10 µl reaction volume in 1.5 ml Eppendorf tube. Ligated samples were transformed in to JM109.

Transformation:
Routinely, 2.5ul of DNA samples were transformed into 50ul of calcium chloride competent cells by heat shock method. Briefly, competent cells were quickly thawed in the palm of the hand. 2.5 ul of DNA was added to 50ul of competent cells and incubated on ice for 30 min. Following ice incubation cells were heat shocked at 42 °C for 90 sec and iced for 2 min. LB Media (800ul) was added to the cells and incubated at 37°C for 40min and 200ul was plated on the LB/Amp plate.

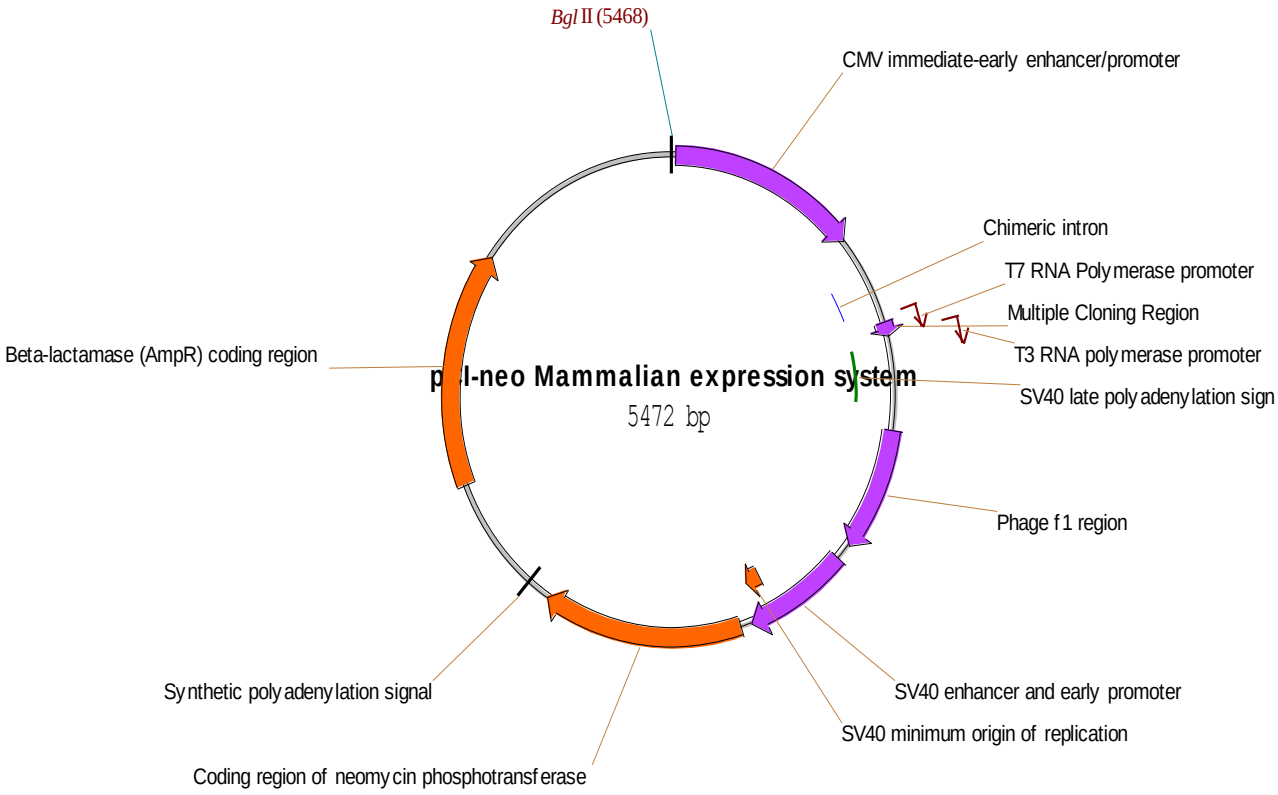
Colony-PCR:
Colony-PCR on recombinant clones was carried out according to the protocols given by sambrook and tussle (Sambrok russel). Reaction was often carried out in 20ul reaction volumes using a single bacterial colony as a template, 50 pmol of O-X-Scr primers in the presence of 200mM dNTPs, 2mM $MgCl_2$ and 1 x concentration of PCR buffer and 1u of Taq. The reaction was carried out in a 0.2 ul PCR tube in a gradient PCR. The PCR program was as shown in the Following PCR amplification PCR samples were separated on 1-2% agarose gel in TAE buffer (pH-8).

Agarose gel electrophoresis:
DNA separation was routinely done in 0.8 to 1% agarose gel in 1 X TAE electrophoresis buffer pH 8.3 (2 mMTris-Acetate/0.05M EDTA). Agarose gels were cast in 1 X TAE buffer containing 0.5 µg /ml of ethidium bromide. DNA samples were mixed with 1/6 volume of 6 X loading dye (Usha Biotech Pvt Ltd, Hyderabad, AP, India) and subjected to electrophoresis under controlled voltage of 5V/cm. Appropriate DNA size markers (1 kb or 100 bp DNA ladder) were run alongside the samples to estimate the size of DNA fragments. The DNA was visualized in an UV transilluminator and gel documentation system (syngene).

RESULTS

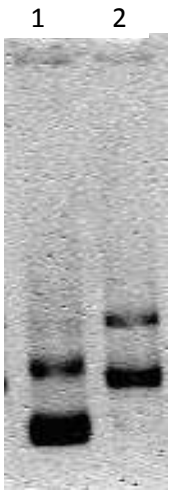
Preparation of pCI-Neo-BglII/CIAP back bone vector:
PCI-Neo mammalian expression vector (Figure -1) consists of a cytomegalovirus promoter (CMV) to drive the expression of Gene of Interest (GOI). The CMV promoter-MCS-PolyA

Figure -1: pCI-Neo-mammalian expression vector



makes the complete transcriptional unit. The plasmid also encodes of neomycin resistance gene which helps in the selection for stable integrants. For the development of double gene mammalian expression plasmid I preferred to clone SV40-MCS-PolyA form pSI in to BglII site. BglII is a unique site and cloning of SV40-MCS-PolyA in to BlgII site will not affect any other functions of the plasmid (Figure -1).

Figure -2: pCI and pSI plasmid



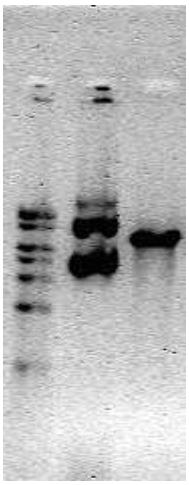
For the creation of pCI-Neo-BglII back bone the plasmid was purified using Usha Biotech Plasmid Purification kit (0). The purified plasmid was analyzed on 1% agarose gel in TAE buffer (**Error! Reference source not found.**). The plasmid was found to be of good concentration and quality (

Figure -2). The two bands in lane 2 represent relaxed and supercoiled state of plasmid.

pCI-Neo-mammalian expression vector was digested with 1u of BglII restriction endonuclease (**Error! Reference source not found.**). The linear plasmid was then dephosphorylated using calf intestinal alkaline phosphatase (CIAP) (**Error! Reference source not found.**). The BglII digested and CIAP treated plasmid was analyzed on 1% agarose gel in TAE buffer (**Error! Reference source not found.**). The gel picture (Figure 3) showed single band between 5 and 7kb indicating that the plasmid is of the right size which is 5472bp.

pCI-Neo-mammalian expression vector encodes for (a) CMV Promoter for transcription of Gene of interest (GOI) in mammalian cells, (b) MCS to clone the GOI, (c) Poly A, (d) Beta lactamase gene for ampicillin selection in bacteria, (e) Neomycin resistance gene for selection in mammalian cells, (f) Bacterial Origin of replication.

Figure 3: pCI digested with BglII and CIAP treatment



pCI and pSI are mammalian expression vectors. pCI consists of CMV promoter to drive the expression of recombinant protein of interest and pSI consists of SV40 promoter to drive the expression of recombinant protein of interest. pSI and pCI plasmids were purified using Usha Biotech Plasmid Purification Kits. Lane 1: (pSI) and Lane 2 (pCI).

Isolation of SV40-MCS-PolyA transcriptional unit from pSI plasmid:

pSI mammalian expression vector consists of SV40 promoter to drive the expression of GOI. The expression plasmid consists of SV40-MCS-Poly A which comprise of the complete transcriptional unit. For the development of a double gene expression vector I preferred to clone SV40-MCS-PolyA in to BglII site of pCI. pSI consists of BglII and BamHI sites on either ends of SV40-MCS-PolyA. Both BglII and BamHI are compatible with BglII ends making it feasible to clone BglII-SV40-MCS-PolyA-BamHI fragment in to BglII site of pCI-neo-mammalian expression vector.

For the isolation of SV40-MCS-PolyA from pSI. The plasmid was purified using Usha biotech plasmid purification kit (0). The purified plasmid was analyzed on 1% agarose gel in TAE buffer. The plasmid was found to be of good quality and of good concentration. The presence of two bands represents the presence of both supercoiled and relaxed forms.

pSI was first digested with BglII (**Error! Reference source not found.**). The BglII digested plasmid purified using desalting kit (0). BglII digested pSI was then digested with BamHI (**Error! Reference source not found.**). The double digested plasmid was separated on 1% TAE buffer (**Error! Reference source not found.**). The presence of 974 bp fragment in the gel indicates the release of SV40-MCS-PolyA fragment. BglII-SV40-MCS-PolyA-BamHI was gel extracted (**Error! Reference source not found.**) and the extracted samples were analyzed on 1% agarose gel (**Error! Reference source not found.**). Gel extracted sample was found to have the 974 bp fragment.

Ligation of pCI-neo-BglII/CIAP with BglII-SV40-MCS-PolyA-BamHI:

SV40-MCS-PolyA isolated in section 00 was ligated with pCI-Neo-BglII/CIAP prepared. Ligation was as described in methods 0. In these ligation reactions sample 1 act as control for vector relegation. And Sample 2 is for the construction of double gene vector.

Ligated samples were transformed in to JM109 competent cells (0) and plated on to LB/Amp plates. Eighteen hours post plating plates were observed for the presence of transformed colonies. The results of transformation were shown in **Error! Reference source not found.**. Only 2 colonies survived in Vector+Insert transformed samples. The two colonies might be carrying the double gene expression vector.

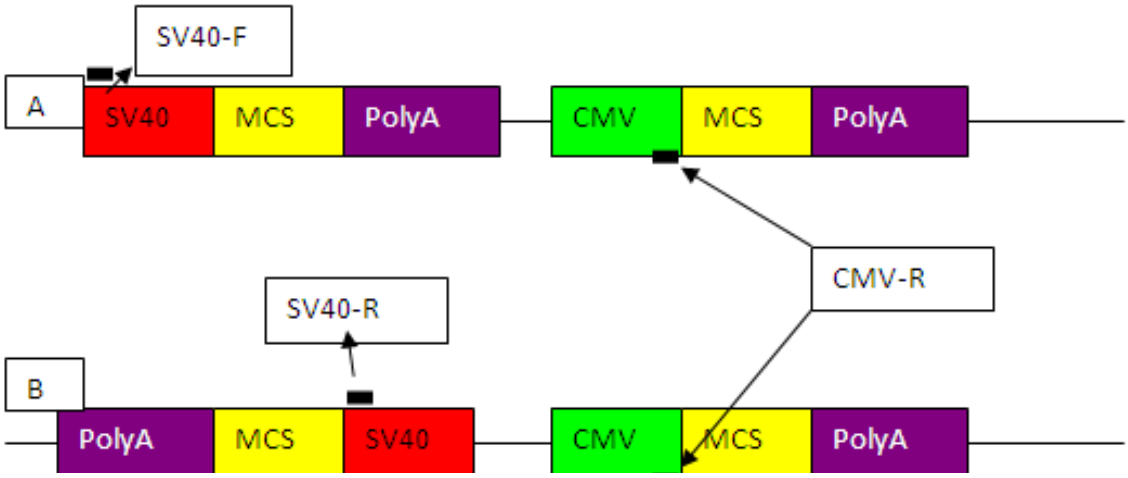
Screening of recombinant clones:

Among the two clones one clone was analyzed for the presence of double gene expression vector using colony PCR using two sets of primers (0). SV40-F and CMV-R will amplify

the orientation shown in **Error! Reference source not found.**-A gives a fragment of the size of 1724bp; However, SV40-R and CMV-R will amplify the orientation as shown in **Error! Reference source not found.**-B and gives a fragment of the size of 1169bp. Both the primers sets can no amplify pCI-neo-mammalian expression vector and pSI mammalian

Traditionally, monoclonal antibodies are expressing co-transfecting light and heavy chin coding plasmids [Johnston k *et al* 2000]. However, this often results in low expression or lack of expression monoclonal antibody. The low or lack of expression is due to the lack of eqimolar concentration of the plasmid in transfected cells or lack of expression of light or

Figure-4. Possible orientation of SV40-MCS-PolyA following ligation in to pCI neo-mammalian expression vector



samples were analyzed on 1% agarose gel in TAE buffer (**Error! Reference source not found.**). SV40-F and CMV-R amplified a fragment of the length of 1724bp indicating the presence of both the transcriptional units and the orientation as shown in **Error! Reference source not found.**.

DISCUSSION

Plasmids are quiet valuable tools in both basic research and applied sciences. Hundreds of plasmids have been designed over the past two decades for various purposes. Mammalian expression plasmids are one among there which facilitate the expression of recombinant protein in mammalian cells. Monoclonal antibodies are the fastest growing therapeutic in the biopharmaceutical industry. However there are complex proteins and require correct post translational modification for their functionality. These are often expressed in mammalian cells (CHO, NSO).

overcome such problems. (a) Use of di-cistronic expression vector, where both light china and heavy chain are linked by an internal ribosomal entry sequences (IRES) on a single plasmid [Rucker.p et al 1997] (b) double gene expression vector, where the plasmid consists of two transcriptional unit to express light and heavy chain form the same plasmid [Susn.k*et al* 2002].

In the current project I have developed a double gene expression vector by cloning SV40-MCS-PolyA from pSI mammalian expression plasmid into pCI neo mammalian expression plasmid. The pUB-C-S double gene expression plasmid consists of two transcriptional units which facilitates the expression of both light chain and heavy chain of monoclonal antibodies from the same plasmid. However, the expression of one gene is regulated by SV40 promoter where as the expression of the other gene is regulated by CMV promoter. The use of different promoters

in our plasmid will minimize the competition for transcriptional machinery.

The pUB-C-S plasmid developed in the study has got both the transcriptional unit in tandem the transcriptional units were arranged had to tail (SV40-MCS-PolyA – CMV-MCS-PolyA). The transcriptional units also contain an intronic sequence next to the promoter to increase the stability of mRNA there by increasing the recombinant protein productivity.

CONCLUSION

A double gene expression vector was developed by cloning SV40-MCS-PolyA in to BglII site of pCI-neo-mammalian expression vector. The presence of insert and its orientation was analyzed by colony PCR. The double gene expression vector consists of two transcriptional units (a) CMV-MCS-PolyA and (b) SV40-MCS-PolyA. Both the transcriptional units are in tandem. SV40-MCS-PolyA-CMV-MCS-PolyA. The double gene expression vector can be used to express both light chain and heavy chain of monoclonal antibodies from the same plasmids there by overcomes the problems that arise do to co-transfection methods.

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