ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 4 (4): 284-287, 2005
ISSN 1682-296X

Site Directed Mutagenesis of V2 Vasopressin Receptor and its Cloning Using PGEM3Z Vector

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Abstract: The present study was conducted to create mutations in this motif for further investigation of its function. By using nested PCR two mutations were produced, which would translate to DRY and DKH. The PCR products as well as the PGEM3Z vector were digested using NcoI and EcoRI restriction enzymes and were ligated and transformed to *E. coli* HB101 cells. The obtained colonies were analyzed for the presence of the inserts using suitable restriction enzymes. The obtained plasmids have the advantage of having restriction sites, which would not interfere with further cloning and expression of this receptor using mammalian expression vectors.

Key words: Vasopression, V2 receptor, PGEM3Z vector, cloning, DRY motif

INTRODUCTION

Vasopressin plays an important role in water reabsorption from the collecting ducts of the kidney by activating V2 receptor[1,2]. Certain mutations in this receptor alter its function and can result in a disease called congenital nephrogenic diabetes insipidus[3] and also can provide important information on the receptor structure–function relationship. Among these is a mutation at the DRY motif (DRH in the case of V2 receptor), which has been isolated from the patients having NDI, rendering the receptor nonfunctional[4]. This along with other studies performed on adrenergic[5,6], gonadotropin-releasing hormone[7], histamine[8] and angiotensin receptors[9,10] show that the DRY motif plays an important role in the G-protein coupled receptor expression and function. However, the effects of this motif on V2 receptor function is not well understood and for this purpose we decided to create two mutations in this region (DRY and DKH).

Expression and function of receptors are normally studied in mammalian cell lines using expression vectors. However, these vectors usually are large plasmids and have many restriction sites, thus sometimes it is needed that cloning vectors are used and then the desired clone is transferred to the expression vectors. We also encountered similar problems regarding production and cloning of our mutations in the expression vectors and thus we decided to subclone them in a cloning vector as the first step for the study of the role of these mutations in the V2 receptor function.

MATERIALS AND METHODS

Materials: 10x PCR buffer and DNA Taq polymerase enzyme were from Bio-tools, dNTP was from Eurobio, restriction enzymes were from Rosch company, PGEM3Z, pcDNA-V2R vector, ligase;enzyme, buffer and DNA weight marker were from Fermentase, plasmid preparation kit were from Bio-Rad, gel extraction kit was from QIAGEN and Bio-Rad.

Methods:

Primer design: Using WDNASIS program, a pair of primers was designed for DRY (aspartate-arginine-tyrosine) and DKH (aspartate-tyrosine-histidine) mutants. Primer sequence for DRY was: sense (tggacaagcaccgtgccatctg) and anti-sense (cagatggcacggtgcttgtcca), for DKH was: sense (gaccgc**t**accgtgccatctgcc) and anti-sense (ggcagatggcacggtagcggtc), for inner in V2R was: sense (cccagcaacagcagccag) and anti-sense (tcatcctcacagtcttgg) and for outer in V2R was: sense (ccaccacttccgctgtgc) and anti-sense (acccaacagctcctcacg).

Nested PCR: A mixture of cDNA of V2 receptor (as template), 10x PCR buffer, dNTP, 1 DNA Taq polymerase, of each sense DRY with anti-sense outer in V2R primers in one eppendorf tube and anti-sense DRY with sense outer in V2R primers in another tube (with total volume 50 μL) was set in Termocycler for preparation PCR products (first PCR). Similar mixture was prepared for DKH mutant. Protocol of PCR was set as following: 1 cycle

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(94°C for 5 min), 33 cycles (94°C for 1 min, 55°C for 2 min, 72°C for 2 min) and 1 cycle (72°C for 20 min). The PCR products were run on gel (1%) and detected their size. In second (nested) PCR, a mixture of 1 the products of first PCR (as template), of 10x buffer of PCR, dNTP, DNA Taq polymerase and of each sense and anti-sense inner primers in V2R (with total volume 50 μL) was set as above.

Gel electrophoresis: The PCR product was run on a 1% agarose gel containing ethidium bromide and then was visualized and photographed using a transluminator and gel documentation system.

Digestion: All digestions were performed using the suitable restriction enzymes at 37°C for 1 h.

Gel extraction: A DNA extraction kit was utilized for isolation of the DNA bands from the agarose gels.

Ligation: The vector and insert (molar ratio of 1/3, respectively), DNA ligase and its buffer were added at a final concentration of 15 μL and were incubated at 16°C overnight.

Transformation: The product of ligation was transformed using HB101 competent cells. The heat shock was performed for 1 min at 42°C. The cells were spread over LB plates containing ampicillin and were incubated overnight at 37°C[11].

Plasmid preparation: The obtained colonies were cultured in LB medium at 37°C overnight. Then plasmids were prepared using alkaline lysis method[11].

RESULTS

By using the external, internal and mutant primers, the nested PCR was carried and the products of the first and second (nested) PCR are shown in Fig. 1 and 2. The second PCR containing the desired mutations were used for the digestion and subcloning into the PGEM3Z vector.

To obtain a PGEM3Z vector having the wild type receptor, the V2 receptor was digested out of the pcDNA3 plasmid using XbaI and EcoRI restriction enzymes (Fig. 3). The same digestion was performed on PGEM3Z plasmid and the wild type V2R was cloned into the PGEM3Z vector (Fig. 4).

The PGEM3Z vector having the wild type V2 receptor as well as the nested PCR products were cut by NheI and NcoI enzymes for obtaining vectors and inserts (Fig. 5).

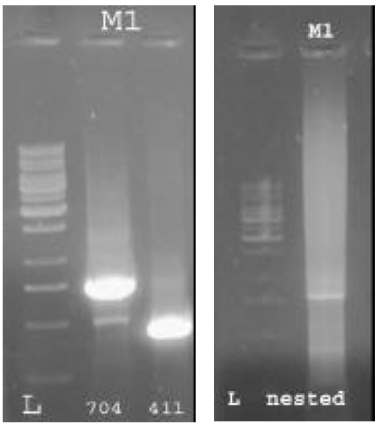

Fig. 1: First (left) and second or nested (right) PCR of DRY mutant (M1) at V2 receptor: Ladder or DND marker is abbreviated as L. In first PCR of sense primer of DRY and anti-sense of outer primer in V2R was produced sharp and dense band in 704 bp size and PCR of anti-sense primer of DRY and sense of outer primer in V2R was produced sharp and dense band in 411 bp size. In second PCR of sense and anti-sense of inner primer, band was detected with 760 bp size; band was detected with 760 bp size

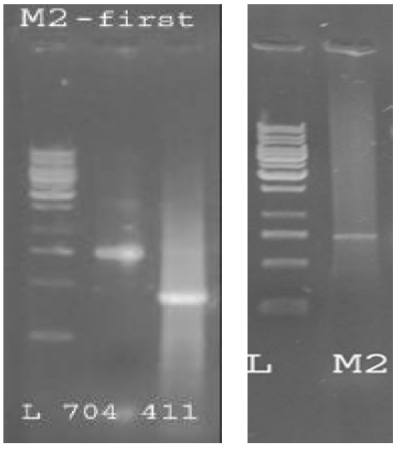

Fig. 2: First (left) and nested (right) PCR of DKH mutant (M2) at V2 receptor. Ladder or DND marker is abbreviated as L. In first PCR of sense primer of DKH and anti-sense of outer primer in V2R was produced sharp and dense band in 704 bp size and PCR of anti-sense primer of DKH and sense of outer primer in V2R was produced sharp and dense band in 411 bp size. In second PCR of sense and anti-sense of inner primer, band was detected with 760 bp size

Fig. 3: Digestion of pcDNA3 by EcoRI and XbaI. Band of V2R have 1200 bp size, which is cut from pcDNA3

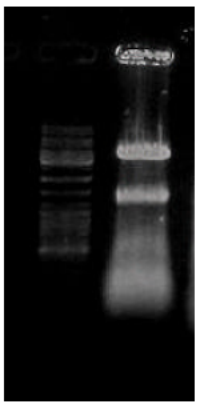

Fig. 4: PGEM3Z containing cDNA V2R digested by EcoRI and XbaI (L: DNA marker). Two bands was detected; 2700 bp band at above of gel, which is PGEM3z and 1200 bp band that is V2R

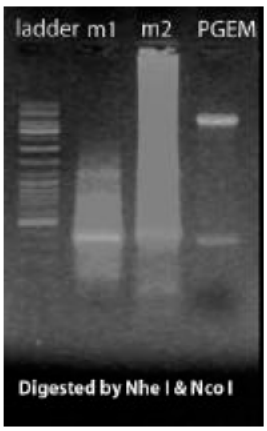

Fig. 5: PGEM3Z and mutants (m1: DRY, m2: DKH) digested by NheI and NcoI enzymes. The band that are showed by arrows, have 342 bp size. These bands of m1 and m2 was extracted from gel and used as inserts

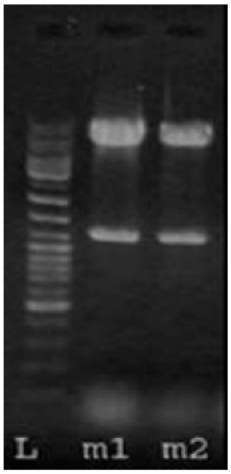

Fig. 6: PGEM3Z containing mutants (m1: DRY, m2: DKH) digested by EcoRI and XbaI and produced 1200 bp bands of V2R

After ligation and transformation, plasmid preparation was performed on the resultant colonies. The presence of the inserts were confirmed by the EcorRI plus Xbal digestion (Fig. 6)

DISCUSSION

We decided to create DRY and DKH mutant DNA in V2 receptor for further study of the DRH motif and its role in the V2 receptor function. As the first step we cloned these mutations into the PGEM3Z cloning vector. This strategy has been utilized by other investigators. For example Balkman *et al.*[12] used the PGEM3Z as cloning vector in their study or Kong *et al.*[13] applied this vector in their research.

The PGEM3Z vector was selected since it gave us the opportunity to cut the minimal length of the V2 receptor and replace it by the mutated PCR fragments. This would minimize the risk of having random mutations created by PCR procedure into the V2 receptor cDNA. For this purpose, NheI and NcoI enzymes were used which cut the V2 receptor cDNA at one site but and do not cut PGEM3Z. Therefore, This strategy allowed us to obtain the desired V2 receptor mutants for further cloning into an expression vector.

ACKNOWLEDGMENTS

This project was carried by MUI grant. We would also like to thank Mrs. Moazen for her assistance in this project.

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