CONSTRUCTION OF AN EXPRESSION VECTOR BASED ON pCDNA3.1(+) EXPRESSING JEMBRANA DISEASE VIRUS ENV-TM SUBUNIT GENE IN EUKARYOTIC SYSTEM

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ABSTRACT

Jembrana disease virus (JDV) is an agent of a highly infectious disease in Bos javanicus, discovered for the first time in Jembrana village, Bali, Indonesia. Its genome, composed of a single-stranded RNA has been entirely sequenced. ENV protein is an interesting antigen of JDV. We have previously isolated env-tm subunit gene by a single-step RT-PCR reaction. We report in this paper the construction of a pcDNA3.1(+) based vector for ENV expression in eukaryotic cells. Env-tm gene was excized from the previous construct pCR2.1-TM by a digestion with BamHI and inserted in BamHI site of pcDNA3.1(+) plasmid. The inserted gene comprises the entire coding sequence, including the C-terminal hydrophobic domain and an additional ATG for correct expression in eukaryotes. 6 out of 7 clones analyzed were positive. This high cloning efficiency was due to correct BamHI cut of pcDNA3.1(+) which was then followed by alkaline phosphatase treatment. Gene orientation was further determined by EcoRI and EcoRV digestions. 2 out of 6 positive clones were in sens orientation while the other 4 were in anti-sens orientation. Clones in sens orientation will be used for protein expression in eukaryotic system.

Keywords: Jembrana Disease Virus (JDV), env-tm gene, pcDNA 3.1 (+)

ABSTRAK


Kata kunci: Virus Jembrana, gen env-tm, pcDNA 3.1 (+).
INTRODUCTION

Jembrana virus is the agent of Jembrana virus disease (JVD), an acute and severe infectious disease of Bali cattle (Bos javanicus), reported for the first time in Jembrana village in Bali, Indonesia (Wilcox et al., 1992). Presently, Jembrana disease has also been detected in other areas of Indonesia, like Lampung (Sumatra), West Sumatra and East Java provinces (Hartaningsih et al., 1993). JVD is not unique to Bos javanicus. It has been reported since that other types of cattle can be infected, namely Bos taurus and crossbred Bali (Bos javanicus x Bos indicus) cattle. The lesions resulted are nevertheless milder when compared to a case fatality rate of about 20% in Bos javanicus (Soeharsono et al., 1995a). Viral particles can be detected in saliva and milk during the acute phase and the titer of infectious virus in blood is high. Known direct transmission of the disease occurs by conjunctival, intranasal or oral routes (Soeharsono et al., 1995b). Extremely high circulating viraemia was observed during the febrile phase in experimentally infected animals and many JVD-infected tissues were demonstrated early in the disease course. The most infected organ is spleen but other organs are also highly infected, i.e. lymph nodes, lungs, bone marrow, liver and kidney (Chadwick et al., 1998).

Antigenic cross-reactivity between elements of JVD and the previously identified bovine lentivirus designated bovine immunodeficiency virus (BIV) suggests that JVD is a lentivirus (Wilcox et al., 1995). The JVD genome has now been entirely sequenced. It is composed of a single-stranded RNA, 7,732 nucleotides in length (Chadwick et al., 1995b). Sequence comparison clearly established that JVD is actually a lentivirus, exhibiting retrovirus characteristics (Wilcox et al., 1995a, 1995b). Though closely related, significant genomic differences were found between JVD and BIV which may be related to the differences observed in their respective pathogenicity (Chadwick et al., 1995b).

JVD has been reported to be also related to human immunodeficiency virus (HIV). Some of its regulatory elements can functionally substitute for those of HIV (Chen et al., 2000). For this reason JVD-based vectors may constitute a safe vector-mediated gene transfer, more readily acceptable than those from HIV for human gene therapy (Metharam et al., 2000).

ENV protein of JVD is an interesting antigen. Env gene is expressed as a precursor protein that is further matured into two subunits, i.e. SU (surface) and TM (transmembrane) proteins. In this paper, we describe the cloning of the entire coding sequence of env-tm subunit gene, including the sequence encoding for the C-terminal hydrophobic domain, in the eukaryotic expression vector pcDNA3.1(+).

MATERIALS AND METHODS

Isolation of env-tm gene

The isolation of env-tm gene has reported in our previous work (Asmarani Kusumawati et al., 2002). Briefly env-tm gene was isolated using a single-step RT-PCR reaction and cloned in pCR2.1-TOPO using topoisomerase reaction methodology. Env-tm gene was excised from the resulting positive clone, called pCR-TM, by BamHI digestion as follows. pCR-TM plasmid was prepared from recombinant E. coli TOP10. A mixture of pCR-TM-positive recombinant bacteria were cultured in 20 ml of LB-ampicillin, at 37°C, for one night. Bacteria were harvested by centrifugation (Sorvall, 3,000 rpm, 4°C, 15 min). Plasmid was purified using a miniprep plasmid kit (Roche), according to the instruction manual. 5 µg of plasmid was digested with 15 units of BamHI in 40 µl of adequate buffer, for 1 h 30 min, at 37°C. 2 µL of the digestion products were analysed by electrophoresis on 0.8% agarose gel. The env-tm gene, about 1.1 kb in length, was purified by electrophoresis on a 0.8% agarose gel and eluted using Gene Clean II kit (BIO101) (glass milk-based procedure) then quantified.

Preparation of pcDNA3.1 vector

Insertion of env-tm gene was done in BamHI site of pcDNA3.1(+) (+). 5 µg of pcDNA3.1(+) (Invitrogen) was digested with 15 units of BamHI (Roche) in 40 µl of adequate buffer at 37°C for 2h 30 min, followed by a treatment with alkaline phosphatase, by adding 20 µL phosphatase buffer, 1 unit alkaline phosphatase and incubation at 37°C for 1 h 30 min. Linearized and dephosphorylated pcDNA3.1(+) was purified by electrophoresis on a 0.8% agarose gel and eluted using Gene Clean II kit (BIO101) (glass milk-based procedure) then quantified.

Construction of pcDNA3.1-env-tm

Ligation:

Insertion of env-tm gene was carried out by ligation of the purified gene to BamHI-digested and dephosphorylated pcDNA3.1(+) (+). Ligation was done in 20 µL of buffer with 200 units of ligase (Biolabs), at 16°C, OVN. The amount of vector (5.4 kb in length) used was 50 ng while the env-tm gene added was about 50 ng. Considering the length of the gene, i.e. about 1.1 kb, the ratio of insert-vector was so about 5:1.

Bacterial transformation:

Ligation products were transfected in E. coli DH5 using the TSS methodology (Asmarani Kusumawati et al., 2002). Briefly, TSS-competent bacteria were obtained by 10 times-concentrating fresh exponential phase bacterial culture (OD600 around 0.6) in LB containing 10% PEG 6,000 (w/v), 5% DMSO (v/v) and 35 mM MgCl2, 0.2 ml competent bacteria were mixed to half of the ligation products, let for 30 min in ice then heat-shocked at 42°C for 90 sec and let in ice again for

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some minutes. 0.5 ml of SOC were added and bacteria were incubated with agitation at 37°C, for 30 min. Different amounts of transformation were spread on ampicillin-agar plate and cultured for OVN, at 37°C. Individual bacterial colonies were cultured in 5 ml LB at 37°C for one night. Bacteria were then harvested by centrifugation at 4,000 rpm (in Sorvall centrifuge), for 15 min, at 4°C.

**Plasmid preparation**

Plasmids were extracted using alkaline lysis method. Briefly, bacterial pellets were dissolved in 0.3 ml buffer 1 (50 mM Tris-HCl, pH 7.5, 10 mM EDTA) and incubated for 5 min at room temperature. 0.3 ml of buffer 2 (0.2 M NaOH, 1% SDS) was then added and the solution mixed without vortexing. Finally, 0.3 ml of buffer 3 (2.55 M potassium acetate, pH 4.8) was added and the solution mixed without vortexing then centrifuged in a minicentrifuge for 15 min at maximal speed (13,000 rpm) at room temperature. The supernatant was then precipitated by addition of 0.7 ml isopropanol and centrifugation (minicentrifuge, 13,000 rpm, 15 min, room temperature). The pellet was washed with 70% ethanol then slightly dried. It was then dissolved in 50 μL 10 mM Tris-HCl pH 7.5 and 0.5 mg/mL RNAses and incubated at 37°C for 30 min.

**Clone analysis**

Clones were analyzed by *Bam*HI digestion. Digestion was carried out in 20 μL of buffer, containing 2.5-5.0 μL of plasmid solution, 5 units enzyme, at 37°C, for 1 h 30 min. Digestion products were analyzed by electrophoresis on a 0.8% agarose gel.

**RESULTS AND DISCUSSION**

**Isolation of env-tm gene and preparation of cloning vector**

ENV protein is an important antigen of Jembrana disease virus (JDV), the agent of acute infectious disease of Bali cattle (*Bos javanicus*). It is composed of 2 subunits i.e. SU (surface) and TM (transmembrane) proteins. *Env*-tm subunit gene, about 1.1 kb in length, was excised from our previous construct pCR-TM by *Bam*HI digestion, as described in Materials and Methods. *Env-tm* gene we previously isolated by a one step RT-PCR reaction and cloned in pCR2.1-TOPO plasmid, comprises the entire coding sequence, including the sequence encoding for the C-terminal hydrophobic domain, an additional initiation codon ATG upstream the gene and a new site for *Bam*HI upstream and downstream the gene, allowing further cloning in expression vectors. Figure 1 shows DNA fragments resulting from *Bam*HI digestion of pCR-TM. As seen, a fragment of about 1.1 kb corresponding to *env-tm* gene length was obtained, in addition to a fragment of 3.9 kb (pCR2.1-TOPO). The 1.1 kb fragment was excized from the gel and purified.

![Fig 1](image_url) Insert corresponding to *env-tm* coding sequences were excised by enzyme *Bam*HI-EcoRI.

Figure 2 shows pcDNA3.1(+) preparation for *env-tm* gene cloning. Shown in this figure analysis of *Bam*HI digestion and analysis of linearized vector after purification. Electrophoretic analysis showed that only a single DNA fragment was obtained. It corresponded to the linearized pcDNA3.1(+), i.e. 5.4 kb in length. Obtaining correct linearized vector was very important as cloning efficiency depends on complete vector digestion. Isolation of linearized cloning vector also prevented contamination of non digested vector which will give rise to negative clones. *Bam*HI digestion of pcDNA3.1(+) was followed by alkaline phosphatase treatment which dephosphorylates the 5'-end so that plasmid self-ligation will not occur. This will enhance the obtention of positive clones as only bacterial transformants harvesting a plasmid with an insert will grow in ampicilline-containing medium.

![Fig 2](image_url) Analysis of digested vector pcDNA 3.1 (+) were excised by enzym *Bam*HI.

**Construction of pcDNA-TM plasmid for env-tm expression in eukaryotic system**

Previous cloning of *env-tm* gene in pCR2.1-TOPO plasmid using topoisomerase methodology (Asmarani Kusumawati et al., 2002) allowed us to easily and rapidly isolate *env-tm* gene. This system enables clone determination solely by the coloration of bacterial colonies and our conception of primers used in RT-PCR reaction made easy gene preparation for cloning in pcDNA3.1(+) Cloning in pcDNA3.1(+) plasmid was carried out in *Bam*HI site, as described in Materials and Methods. Figure 3 shows bacterial...
transformation with half of the ligation products which represented 25 ng of plasmid. As seen, more than 1,500-2,000 ampicillin-resistant bacterial colonies were obtained. They represented recombinant bacteria harvesting pcDNA3.1(+) plasmid either with or without env-tm gene insertion. 7 bacterial colonies were cultured and plasmids prepared by alkaline lysis method. Positive clones were determined by BamHI digestion. 6 out of 7 clones analyzed were positive, giving rise to 2 DNA fragments of 5.4 kb (pcDNA3.1(+)) and of about 1.1 kb (env-tm gene) respectively (Fig. 4). This high cloning efficiency was due to correct insert preparation on one hand and on the other hand to correct digestion of pcDNA3.1(+) followed by alkaline phosphatase treatment. It is possible to by-pass direct cloning of RT-PCR products in pCR2.1-TOPO and to use the RT-PCR products for cloning in pcDNA3.1(+). This procedure seems to be more rapid. In fact, it is not the case because correct digestion of amplification products is difficult to realize and digestion can not be easily controlled. Therefore cloning will be much less efficient and more time is required. Excision of env-tm gene from pCR-TM construct can be controlled therefore correct digestion can be easily established. The high cloning efficiency was also attained because the cloning vector was treated

As env-tm gene was inserted in a single site, insertion occurs in sense as well as in anti-sense orientation with regard to the CMV promoter of pcDNA3.1(+). Orientation was determined by EcoRI and EcoRV digestion. Table I gives the expected number and length of DNA fragments obtained by these digestions.

All the 7 clones already analyzed with BamHI, were digested with EcoRI and EcoRV, as described in Materials and Methods. Figure 5 shows EcoRI digestion analysis. It confirmed that clone 2 was actually a negative clone, giving rise to a single fragment corresponding to pcDNA3.1(+) length. As seen in this figure, 2 out of 6 positive clones gave rise to two fragments of around 5.7 kb and around 0.75 kb.

Fig. 5 Analysis of constructs base on pcDNA-TM were excised by enzym EcoRI by electrophoresis on agarose gel.

Fig. 6 Analysis of constructs base on pcDNA-TM were excised by enzym EcoRV by electrophoresis on agarose gel.
### Table I: Orientation determination of pCDNA-TM constructs

<table>
<thead>
<tr>
<th></th>
<th>pCDNA3.1(+)</th>
<th>pCDNA-TM/Sens</th>
<th>pCDNA-TM/AntiSens</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI</td>
<td>5,400</td>
<td>5,705; 755</td>
<td>6,118; 345</td>
</tr>
<tr>
<td>EcoRV</td>
<td>5,400</td>
<td>6,403; 60</td>
<td>5,400; 1,063</td>
</tr>
<tr>
<td>BamHI</td>
<td>5,400</td>
<td>5,400; 1,063</td>
<td>5,400; 1,063</td>
</tr>
</tbody>
</table>

They are positive clones in sense orientation. The other 4 clones, giving rise to fragments around 6.0 kb and less than 0.4 kb, were positive clones in anti-sense orientation. Analysis by EcoRV confirmed that clones 2 and 5 are in sense orientation and that clones 1, 3, 4 and 6 are in anti-sense orientation (Fig. 6). Figure 7 represents the map of env-tm gene construct in pCDNA3.1(+) we called pCDNA-TM.

The work described in this paper constitutes the first step towards env-tm subunit gene expression in eukaryotic system. The gene we inserted in the eukaryotic expression vector pCDNA3.1(+) comprises the entire coding sequence, including the sequence encoding for the highly hydrophobic C-terminal domain. The gene also contains an additional initiation codon ATG upstream of the first codon GCC (coding for Ala) of env-tm gene. Due to eukaryotic translation mechanism, the ATG we introduced was intended for correct protein expression in eukaryotic cells.

![Figure 7 The map of pCDNA-TM](image)

**REFERENCES**


