

Construction of an Eukaryotic Expression Vector Encoding Herpes Simplex Virus Type 2 Glycoprotein D and *In Vitro* Expression of the Desired Protein

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Summary

To construct of an eukaryotic expression vector encoding herpes simplex virus type 2 (HSV-2) glycoprotein D (gD₂), an Iranian isolate of HSV-2 was propagated in HeLa cell line and its DNA was extracted and used as template in polymerase chain reactions (PCR), to amplify gD₂ gene. Primers were designed and the restriction enzyme sites for *EcoRI* and *XhoI* were considered at their 5' ends respectively. The PCR product was confirmed by restriction enzyme analysis, cloned into a cloning vector (pBsc) and then sequenced. The fragment encoding gD₂ was obtained by digestion using appropriate enzymes and extracted on agarose gel using a commercial kit. The gene of interest was subcloned in the named sites of an eukaryotic expression vector (pcDNA₃) to construct pcDNA-gD₂. An endotoxin free column was applied to prepare pure pcDNA-gD₂, which was transfected into mammalian cells using lipofectamine. Protein expression was confirmed using indirect immunofluorescent test. The results indicated that the construct expresses in mammalian cell lines effectively and it can be used as a DNA vaccine in animal models.

Key words: HSV-2, DNA vaccine, glycoprotein D, transfection

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Introduction

Herpes simplex virus type 2 (HSV-2) infections constitute a serious world-wide public health problem (Strasser *et al* 2000). Antiviral drug therapy shortens the severity and duration of lesions and reduces recurrences; however it could not be able to prevent spreading of infection among uninfected individuals, so there is a need for an effective vaccine for prophylaxis. Many approaches to HSV vaccines have been evaluated including killed whole virus, live attenuated, subunit, vectored and DNA vaccines. Because of some advantages such as safety, less expensive and eliminating the purification of recombinant proteins, DNA vaccination is an attractive approach to HSV vaccine development (Meseda *et al* 2002). In addition, DNA-based immunization elicits a significant cellular immune response and cytotoxic T lymphocytes (CTLs) as well as humoral immunity to the expressed antigens (Higgins *et al* 2000). It has been shown that using plasmid DNAs encoding HSV proteins could evoke a protective immunity in animal models. HSV-2 glycoprotein D (gD₂) is an essential protein that implicated in virus infectivity. Herpes simplex virus glycoproteins B and D are essential for infectivity and attractive choices for DNA vaccines as they are targets for both humoral and cellular immunity (Flo *et al* 2000).

The aim of the present study was to construct an eukaryotic expression vector containing HSV-gD₂ obtained from an Iranian isolate. This product could be used as a DNA vaccine alone or in combination with recombinant gD₂ for immunization of animal models and evaluation their efficacy.

Materials and Methods

Cells and Virus. The HeLa cell line and Baby Hamster kidney (BHK-T7) were kindly provided by Dr. A. Shafiy. (Razi Vaccine & Serum Research Institute, Karaj, Iran) and Dr. T. Bamdad (Tarbiat Modarres University, Tehran, Iran). Cos-7 cell line was purchased from Cell Bank (Pasture Institute of Iran). Cells were grown and

maintained in Dulbecco's Minimum Essential Medium (DMEM, Gibco) supplemented with 7-10% fetal bovine serum (Gibco), 0.2% sodium bicarbonate (Sigma), 100U/ml penicillin and 100µg/ml streptomycin at 37°C. Solid and liquid Luria Bertani media (LB) were purchased from Mirmedia and supplemented with 100mg/ml ampicillin whenever required. HSV-2 was isolated in our laboratory previously and confirmed by restriction enzyme digestion analysis (Tafreshi *et al* 2004) and by direct IF test using monoclonal anti-HSV-2 antibody (IMAGEN, DAKO) conjugated with fluorescein isothiocyanate (FITC). The virus was propagated in HeLa cell line and harvested by freezing and thawing once at maximum cytopathic effect (CPE). Then centrifuged at 3000rpm for 10min and supernatant was aliquoted and stocked at -80°C before use. The virus was titrated using HeLa monolayer cells on 96-well microplate and reported as TCID₅₀ (Lennette *et al* 1995).

Bacterial strain and plasmids. The *E.coli* DH5α was used as host during the cloning experiments and for propagation of plasmids. The prokaryotic cloning vector, pBluescript (Stratagene) and the eukaryotic expression vector, PCDNA₃ (Invitrogen) were used for cloning of HSV-2 gD gene.

Polymerase chain reaction. HSV-2 DNA was extracted from infected HeLa cells according to standard protocol (Sambrook & Russell 2001). The US₆ open reading frame encoding gD₂ was amplified from genomic HSV-2 DNA using synthetic oligonucleotide primers, gD₂-f: 5'-CTG TGA ATT CCG TAT CAC GGC ATG G-3' and gD₂-r: 5'-GTA CTC GAG AGC GGG GAA ACT CCT C-3' which were deduced from the terminal region of the presented sequences of the HSV-2 gD in GenBank (Acc. No. AF021342K02373U12182). The restriction enzyme sites for *EcoRI* and *XhoI* were considered at 5' ends of the primers respectively as underlined. PCR were done in a total volume of 50µl PCR mixture containing 20mM Tris-Hcl pH8.8, 10mM KCl, 10mM (NH₄)₂ So₄, 2mM MgSo₄, 10mM of each dNTP, 20pmol of each primer, 0.1µg DNA template, 0.5U pfu DNA polymerase (Fermentas),

distilled water to a viral volume of 50µl and covered with 50µl of mineral oil to prevent evaporation. All amplification reactions were performed in a thermocycler (Mastercycler- eppendorf) under the following condition: 3min at 95°C followed by 30 cycle of 1min at 95°C for denaturation, 1min at 65°C for primer annealing and 90sec at 72°C for extension, and a final extension step at 72°C for 5min. Appropriate negative controls were included in parallel to be sure about contamination. Expected PCR product was evaluated through 1% agarose gel stained with ethidium bromide upon preparation. The PCR product was extracted from the gel after confirming by restriction enzyme analysis using *SalI*.

Cloning of gD₂ gene into the cloning vector. The PCR product was cloned into the linearized pBluescript according to the standard protocol (Sambrook & Russell 2001). White colonies that maybe harbor recombinant plasmid were grown and their plasmid were extracted and evaluated by gel electrophoresis onto 1% agarose gel to monitor transformants. The recombinant vector was confirmed using restriction enzyme analysis and was sequenced by MWG Co., (Germany).

Subcloning of the gD₂ into pcDNA₃. The gD₂ gene was subcloned into pcDNA₃ under the control of the CMV immediate early promoter. The eukaryotic expression vector and the recombinant pBluescript were digested with *EcoRI* and *XhoI* and electrophoresed on 1% agarose gel. The gene of interest and linear pcDNA₃ were extracted from the agarose using Nucleospin Extract (Macherey-Nagel). The 1.2kb fragment which encoding gD₂ was inserted into the appropriate sites of the pcDNA₃ vector and ligated using a procedure that was described previously. Transformants were selected on LB agar containing ampicillin, confirmed using gel electrophoresis and restriction enzyme analysis. After confirmation it was named pcDNA₃-gD₂. Maxi preparation of the interest clone was done using an endotoxin free midiprep plasmid isolation kit (Macherey-Nagel).

In vitro evaluation. gD₂ expression in mammalian cells was examined by indirect immunofluorescence test. PcDNA₃-gD₂ was transfected in COS-7 and BHK-T7 cells

using lipofectamine (Invitrogen) according to the manufacturer instruction. After 48h the coverslips were washed three times with PBS ($\text{NaH}_2\text{PO}_4\text{H}_2\text{O}$ 50mM, $\text{Na}_2\text{HPO}_4\text{H}_2\text{O}$ 20mM), then fixed by cold methanol for 10min at -20°C . The cells were washed with cold PBS and reacted with human anti- HSV-2 positive serum, which was diluted to 1:100 in PBS for 1h at 37°C . Cells were washed and the bounded antibodies reacted with fluorescein isothiocyanate-labeled goat antihuman IgG (Biogen) that was diluted 1:200 in PBS for 30min. Finally cells were washed with PBS and mounted with glycerol and viewed under a Ziess Fluorescent microscope.

Results

A clinical isolate of HSV-2 was propagated in HeLa cells and the virus was harvested while maximum CPE was observed and titrated using HeLa cell monolayers on 96-well microplate, calculated through Karber formula and reported as $10^{-4.5}\text{TCID}_{50}/100\mu\text{l}$. The PCR was performed using designed specific primers to obtain the gD_2 gene. The PCR product (Figure 1) was confirmed by digestion with *SalI* that has one cut site in position 740.

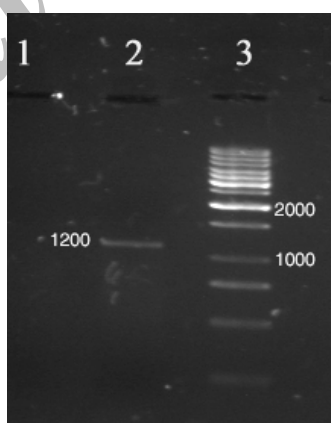


Figure 1. Agarose gel electrophoresis of amplified gD_2 by PCR. Lane 3, DNA size marker (ladder 1kb); lane 1, negative control; lane 2, 1.2kb PCR product

The HSV-2 US6 open-reading frame, encoding the viral gD was cloned into a cloning vector. The recombinant pBluescript (pBsc-gD₂) was confirmed by restriction analysis (Figure 2) and sequencing. Analysis of sequencing was accomplished by chromas software (version 1.45-Australia) and revealed about more than 99% homology with sequences that are presented in GenBank. The nucleotide sequence data was deposited in GenBank database under the accession number 'AY 517492'.

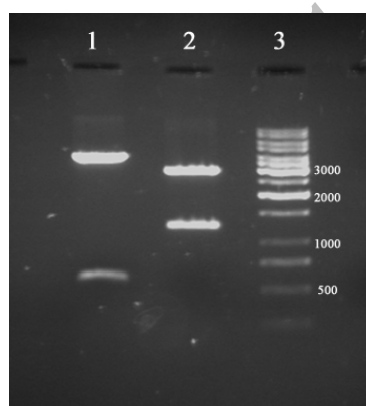


Figure 2. Restriction analysis of pBsc-gD₂. Lane 3, DNA size marker (ladder 1kb); lane 2, pBsc-gD₂ digested with *EcoRI* and *XhoI*; lane 1, pBsc-gD₂ digested with *Sall*

Transient expression of HSV-2 gD₂ was examined by indirect immunofluorescence test by 48h post transfection. Transfected BHK-T7 and COS-7 cells with pcDNA₃-gD₂ are shown in figures 3 and 4, respectively.

Discussion

Herpes simplex virus infection is a major cause of morbidity in humans (Sin *et al* 1999). HSV-2 infects mucocutaneously and causes genital infection. Studies of protective immunity to HSV are confounded by the ability of the virus to establish primary infection and to reactivate in the presence of a demonstrable host immune response, although there are evidences that primary virus infection elicits a

protective response against subsequent re-infection. The complex nature of the virus likely induces multiple immune responses during infection, including both humoral and cellular responses (Nass *et al* 2001).

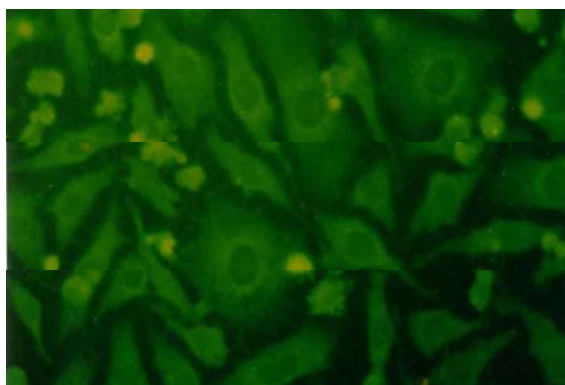


Figure 3. Transfected BHK-T7 with $pcDNA_3$ -gD₂ examined by IFT ($\times 400$).

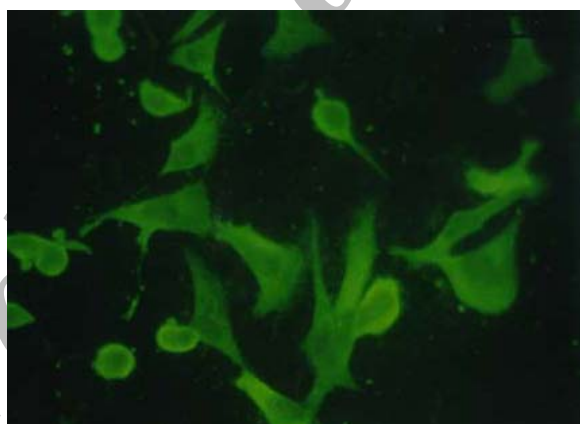


Figure 4. Transfected COS-7 with $pcDNA_3$ -gD₂ examined by IFT ($\times 400$).

Several reports have described the use of plasmid DNAs encoding HSV proteins to evoke a protective immunity in animal models (Manickan *et al* 1995). HSV encodes at least 11 glycoproteins. The initial attachment of HSV with cell surface receptor such as heparansulfate is mediated by gC and/or gB. This is followed by

interaction of gD with a cellular molecule, possibly the mannose-6-phosphate receptor. Then gD and gB, gI and gL act alone or in combination to trigger pH-independent fusion of the viral envelope and the host cell plasma membrane. gD is essential for entry into mammalian cells and has been implicated in cell fusion, super infection restriction and neuroinvasiveness. It is encoded by US6 compartment of HSV genome and its molecular weight is approximately 45kD (Cooper 1994, WuDunn & Spear 1989). This protein is highly conserved and antigenically cross-reactive between HSV-1 and HSV-2. This increased the belief that, gD could function as a preventive vaccine against both types of HSV infection (Sin *et al* 1999).

In the present study, an eukaryotic expression vector containing gD₂ gene was constructed using an Iranian isolate of HSV-2. The virus was propagated in cell line and its DNA was extracted using various protocols. A variety of DNA polymerases could be used in PCR. In this study, Pfu DNA polymerase was used to amplify gD₂ gene. It has 3' to 5' exonuclease activity, so it prevents wrong substitution of nucleotides during amplification reactions and is recommended for cloning of the PCR product. This enzyme creates a blunt-ended PCR product, which is inserted into pBluescript as a cloning vector. The cloning vectors such as pBsc, which has LacZ operon are very usable for direct cloning of blunt-ended PCR product. Insertion of a fragment of foreign DNA into the polycloning site of the plasmid result in production of an useless peptide that is not capable of α -complementation. Bacteria carrying recombinant plasmid form white colonies among blue ones in the presence of the chromogenic X-gal. The development of this simple color test has simplified the identification of recombinants constructed in plasmid vectors. The structure of these recombinants can then be verified by restriction analysis of minipreparation of vector DNA. Moreover, they are preferable because of having an ability to be sequenced using universal primers. Our construct was sequenced using M13 primers by MWG Co., (Germany) and in addition of gD₂ sequence, intact

sequence of gene specific primers was confirmed.

Expression of the HSV-2 gD gene product in mammalian cells was achieved by transfection of pcDNA₃-gD₂ to COS-7 and BHK-T7 cells. The expression vector in this study encodes a signal peptide that directs the nascent gD polypeptide into the endoplasmic reticulum, where protein folding, glycosylation and disulfide bond formation take place. After insertion, the signal peptide is cleaved by a host signal peptidase. The protein continues to be translocated through the secretory pathway but remains associated with the plasma membrane via the transmembrane region (TMR), a hydrophobic region of the molecule that spans the lipid bilayer. The HSV-2 gD molecule is thus exposed on the outer surface of the cell (Higgins *et al* 2000). Consistent with this, fluorescent microscopy (Figures 3 and 4) revealed that cells transfected with this plasmid expresses a protein on the cell surface that is recognized by a HSV-2 polyclonal antibody.

Transfection was done using various methods and the highest efficiency was observed when lipofectamine was used as a transfection reagent. It is considered as a simple and efficient method for transferring of foreign gene to eukaryotic cells. COS-7 has been derived from the African green monkey's kidney that carries integrated copies of the SV40 genome and express biochemically active, SV40-encoded large T antigen. The large T antigen derives replication of the transfected plasmid by activating the SV40 origin of relication. Consequently, COS cells are able to support the replication of any plasmid contains an intact SV40 origin. Transfection of COS cells with recombinant plasmid containing an SV40 origin and a functional unit such as pcDNA₃-gD₂, leads to efficient amplification of the transfecting DNA and an enhanced level of transient expression of the cloned DNA segment (Gluzman 1981). BHK-T7 is a cell clone that stably expresses T7 RNA polymerase and resulted in highly transcription and consequence expression of foreign gene which is placed downstream of T7 promoter (Ito *et al* 2003). Our finding demonstrated that pcDNA₃-gD₂ expresses in both cell lines efficiently.

Considering above results, pcDNA₃-gD₂ can be used as a DNA vaccine in animal models. Genetic adjuvant such as interleukins gene can be used for improving of DNA vaccine efficiency.

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