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Original Article

Expression of Influenza Heamagglutinin Globular Head in Different Eukaryotic Cells

Tavakoli R[1], Fotouhi F[2*], Tavassoti-Kheiri M[2], Farahmand B[2], Saleh M[2], Tabatabaeiyan M[2]

1. Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran.
2. Influenza Research Lab, Pasteur Institute of Iran, Tehran, Iran.

Abstract

Background and Aims: Influenza (flu) is a respiratory infection in mammals and birds. It is caused by an RNA virus in the family Orthomyxoviridae. The virus is divided into three main types. Influenza virus type A is found in a wide variety of bird and mammal species and can undergo major shifts in immunological properties. Hemagglutinin (HA) is an important influenza virus surface antigen that is highly topical in influenza research. In the present study, the gene encoding HA1 protein which includes Hemagglutinin globular head from influenza virus A/Tehran/18/2010 (H1N1) was cloned into a eukaryotic expression plasmid (pCDNA3) and its expression was evaluated in eukaryotic cells.

Materials and Methods: HA1 gene was incised from pFastBacTHc-HA1 by digestion, purified and subcloned into eukaryotic expression vector (pCDNA3). After verification of the cloning fidelity, the recombinant plasmid was transfected into COS-7 and BHK-21 cells, and its expression was detected by RT-PCR.

Results: Restriction endonuclease digestion analysis, colony PCR and DNA sequencing indicated that the recombinant plasmid pCDNA3-HA1 had been constructed successfully. After transfection into eukaryotic cells, the presence of mRNA transcripts was verified by reverse transcriptase-polymerase chain reaction (RT-PCR).

Conclusion: This study is a demonstrated success in the construction of eukaryotic expression plasmid for HA1 thus providing a basis for further probing into mechanism of virus infection and exploring DNA vaccine.

Keywords: Influenza virus; Hemagglutinin; DNA vaccine

Introduction

The 2009 flu pandemic was caused by a new swine-origin influenza A (H1N1) virus which was a reassortment between human H3N2, swine H1N1 and avian H5N1strains. The mixing of new genetic elements in swine can result in the emergence of viruses with pandemic potential in humans. As 2009 H1N1 influenza is a new virus and most people have no or little immunity this virus could cause more infections than are seen with seasonal flu. The virus spread worldwide by human-to-human transmission, causing the World Health Organization to raise its pandemic alert to the highest level 6 (1, 2). Hemagglutinin (HA) is a glycosylated integral membrane protein presented in a homotrimer form on the surface of the virus, which mediates adsorption and penetration of virus during infection, HA binds to sialic acid containing receptors on host cells and induces production of neutralizing antibodies. Anti-HA antibodies block virus attachment to sialic acid reseptors of the host cells and prevent infection (3, 4). Hemagglutinin is the most important

***Corresponding author:** Fatemeh Fotouhi, PhD. Influenza Research Lab, Pasteur Institute of Iran, Tehran, Iran.
Email: fotouhi44@yahoo.com

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surface protein and plays a major role in the determination of host range restriction and virulence. Its rod shape makes it ideal for penetration of the virus into the cytoplasm of the cell it is trying to infect. The penetration allows for the eventual release of the viral nucleocapsids into the cell (5). The HA protein is expressed in the whole form HA0 that is proteolytically cleaved into two subunits, HA1 and HA2, which are held together by a single disulfide bond. The HA1 subunit forms the globular head of the molecule. It contains the receptor binding site and conformationally dependent epitopes to which neutralizing antibodies are primarily raised against (6).

Due to the frequent antigenic drift/shift of influenza virus A, the vaccine needs constant modification. This means that each year a new vaccine needs to be made to match the three viruses predicated to likely infect the world's population. Currently, the vaccine is developed in chicken eggs, which can tack months before a workable vaccine is ready for distribution. This becomes a problem when the virus suddenly mutates and the current vaccine is uneffective (1, 7). For this reason the new generation of vaccines is highly demanded.

Influenza DNA vaccines have been well studied for years. The earliest DNA vaccine development in mice conducted using influenza as a model (8). DNA vaccines are based on delivering of only a portion of virus antigens in the form of DNA which is needed to mount a sufficient immune response to combat infection. Naked DNA is transfected into target cells and pushes the cells to produce the desired proteins. DNA vaccines are an effective way to create immunity because the antigens are produced within host cells and therefore are presented via both MHC-I and MHC-II pathways. DNA vaccines elicit a response in both humoral (antibodies) and cellular (cell mediated) arms of the immune system. Another critical advantage of DNA vaccines is that the antigenic proteins are produced endogenously creating a native, tertiary structure compared to the chemically treated virions used in current flu vaccines which can incur changes in folding subsequently reducing the immunogenicity of the vaccine (2, 9. 10).

In the present study, the gene encoding HA1 from influenza virus A/Tehran/18/2010(H1N1) was cloned into a eukaryotic expression plasmid (pCDNA3) and transfected to COS-7 and BHK-21 cells and eukaryotic expression was determined. This construct could be used as a potent DNA vaccine.

Methods

Construction and identification of pCDNA3-HA1

The HA1 gene sequence (996 bp) has been cloned into pFastBac vector by Yousefi *et al* (11). In this study the HA1 gene sequence was obtained from pFastBac-HA1 by digestion and subcloned into the eukaryotic expression vector, pCDNA3 (Invitrogen, Grand Island, NY), downstream of the CMV promoter.

Both the recombinant plasmid pFastBac-HA1 and pCDNA3 were digested by using EcoRI and XhoI enzymes (Fermentas). Following 1% agarose gel electrophoresis, the 1Kb fragment HA1 gene was excised and recovered using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The target fragment was ligated into linearized pCDNA3 plasmid by using T4 DNA ligase (Fermentas) at 8°C overnight. The ligation product was transformed into competent E.coli top10f ′ through selection of the transformants on ampicillin (100U/ml, Sigma-Aldrich) and tetracycline (10 mg/ml, Sigma-Aldrich) containing agar plate. The potential positive colonies were selected by colony PCR using HA1 specific primers (11). Plasmid DNAs from individual colonies were isolated and purified with plasmid miniprep kit (Fermentas). The positive colonies were identified by digestion using EcoRI and XhoI restriction enzymes. The recombinant plasmids were then sequenced by the sequencing core facility of the CinnaGen Co. (Tehran, Iran) in order to confirm cloning accuracy.

Cell transfection

COS-7 and BHK-21 cell lines were received from the National Cell Bank of Iran (NCBI) affiliated with the Pasteur Institute of Iran,

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cultured and maintained in Dulbecco's modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). Briefly, one day before transfection, cells from confluent culture were transferred to 24 well plate (Nunc, Roskilde, Denmark) containing 10^5 cells per well. The cells were incubated for 24 h at 37°C in a humidified incubator containing 5% CO2 to achieve approximately 70-75% confluency on the day of transfsction. Each well of cells was transfected with 0.8µg of plasmid DNA encoding the HA1 gene using lipofectamin 2000 reagent (Invitrogen, Carlsbad, CA; Cat. No. 11668-027) according to the manufacture's instruction. After 48h incubation at 37◦C, cells were examined by fluorescence microscope.

RT-PCR for confirming expression of pCDNA3-HA1

To determine the presence of specific HA1 mRNA in the COS-7 and BHK-21 cells, RT-PCR was performed at 24 h after transfection. Total RNA was extracted from harvested cells with High Pure Viral RNA Kit (Roche, Germany) and treated with RNase-free DNaseI enzyme (Fermentas) to remove template DNA from RNA preparation. Complementary DNA (cDNA) was synthesized by random hexamer primer (Fermentas) and ReverAidTM M-MuLV Reverse Transcriptase enzyme (Fermentas), and used directly for amplification of target gene by PCR using specific primers. The amplified fragment was visualized by agarose gel electrophoresis.

Results

Identification of recombinant plasmids

The presence of HA1 gene in the recombinant plasmid pCDNA3-HA1 was screened by colony PCR using specific primers (figure 1), and verified by restriction enzyme analysis using a combination of EcoRI and XhoI enzymes. As shown in figure 2, a 700 bp HA1 gene fragment presents in gel electrophoresis. Finally, sequencing of the recombinant pcDNA3 confirmed the correctness of cloning procedure.

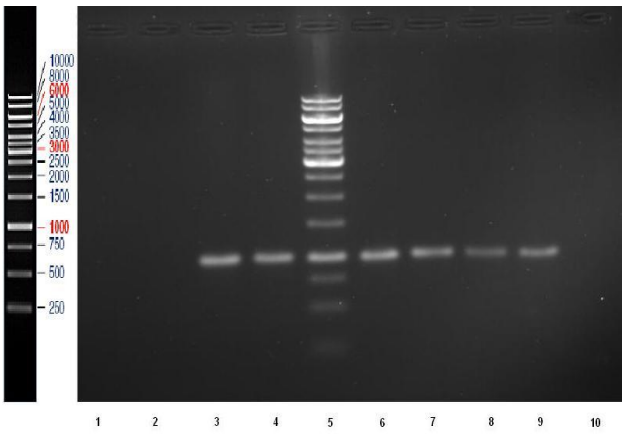

Fig. 1. Results of colony PCR. Lane 1and 2: negative colonies; Lane 3, 4, 6, 7, 8, 9: positive colonies; Lane 10: negative control of PCR; Lane 5: Ladder 1Kb.

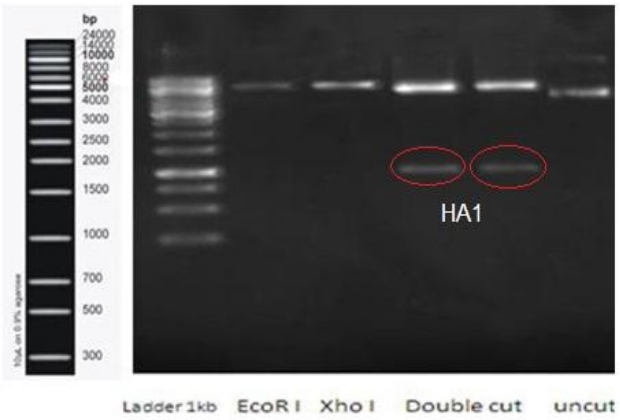

Fig. 2. Restriction enzyme analysis of recombinant plasmid pcDNA3-HA1 with EcoRI/XhoI enzymes resulted in a 1500 bp Fragment.

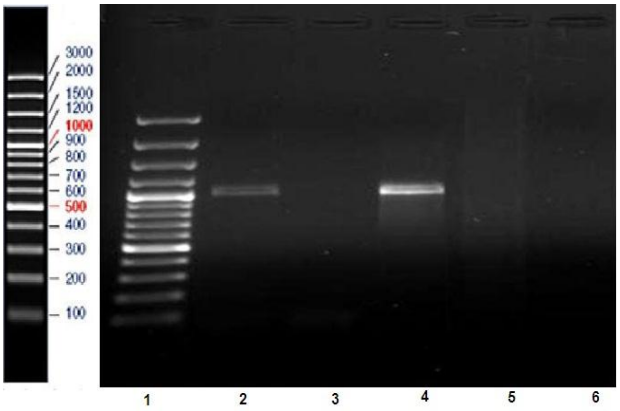

Fig. 3. RT-PCR results from transfected BHK-21 cells. 1- Ladder 100bp, 2- RT-PCR of RNA that was treated with DNaseI, 3- PCR of RNA that was treated with DNaseI, 4- PCR of RNA that was not treated with DNaseI, 5- RT-PCR of RNA from cell control (was not transfected), 6- negative control of PCR.

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Determination of hemagglutinin expression by RT-PCR

After the recombinant plasmid being transfected into COS-7 and BHK-21 cells, the presences of transcript mRNAs were verified by Reverse Transcriptase-Polymerase Chain Reaction (Figure 3). As expected, PCR result of RNA that was not treated with DNaseI, was positive (lane4) which shows contamination of RNA sample with plasmid DNA. While the PCR result of treated RNA sample was negative that indicated correctness of DNaseI function. RT-PCR results from COS-7 (not shown) and BHK-21cells were similar. The presence of 1000bp fragment shows that transcription of hemagglutinin was held on in BHK-21 cells.

Discussion

Genetic vaccines are composed only of DNA or RNA, which is taken up by cells and translated into protein (12). Since there are limited reports on RNA vaccines compared to extensive literature on DNA vaccines, genetic vaccines generally refer to plasmid DNA antigen-expression systems (13). Genetic immunization, also termed DNA or polynucleotide immunization is a novel vaccine technology that developed following the first report that direct injection of plasmid DNA generated the successful expression of the plasmid-encoded antigen in murine muscle cells (2, 14). This unexpectedly successful new method has been described as the "third vaccine revolution" (15, 16) or "the third generation of vaccines" (15, 12). Since its initial development in 1990, this technology has been used to generate humoral and cell mediated immune responses in a wide variety of specific for numerous viral, bacterial and parasitic diseases in a range of animal models, genetic therapies against cancer and autoimmune diseases. In addition, DNA vaccines have become a valuable laboratory tool for a variety of applications ranging from proteomics to understanding the mechanism of antigen presentation, the role of cytokines and the effects of bacterial DNA in the generation of immune responses (13, 17).

Numerous studies of influenza A DNA vaccines have demonstrated that they are capable of inducing humoral and cell mediated immune responses and conferred protection against influenza A viruses in range of species via various delivery routes. In 2006 the results of the first clinical trial of gene expression-based vaccines for influenza HA gene was released and showed the vaccine to be safe and effective in humans (7).

In addition to the receptor binding domain (RBD) in globular head of hemagglutinin, there are several antigenic sites in region that surround the RBD. In the influenza virus A/H1N1, there are five sites that contain Sa, Sb, Ca1, Ca2 and Cb (1, 3). The epitopes in the globular head of hemagglutinin typically elicit strain-specific antibodies because of the hyper variability of this region. On the other hand in 2011, Krause JC et al showed that there is a novel epitope between the receptor-binding pocket and the Ca2 antigenic site on HA which human monoclonal antibody for this region neutralized a broad spectrum of 20th century H1N1 viruses and the 2009 pandemic H1N1 virus. This antibody exposes a new mechanism underlying broad immunity against H1N1 influenza viruses and identifies a conserved epitope that might be incorporated into engineered H1 virus vaccines (18).

The successful construction of HA1-expressing eukaryotic system in this study has not only paved the way for further probing into mechanism of virus infection and exploring DNA vaccine, but also provided a tool for dissecting the targeting site of anti influenza drugs.

To evaluate protein expression in eukaryotic cells, researches were performed by SDS-PAGE, Western blotting and Immunofluorescence assay (IF) using monoclonal antibodies, but the result was not good enough to be reported. It can be due to the very low expression of hemagglutinin gene in COS-7 and BHK-21 cells and need to be gene codon optimized. Codon optimization is performed as a way to increase protein expression in a particular cell line or organism by changing specific codons to those preferred by that host species. In 2010, Tenbusch. M *et*

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al were showed that expression of HA from the wildtype sequence of the novel swine origin H1N1 influenza virus was not visible, but the H1N1 hemagglutinin was well expressed from the codon-optimized sequence (6).

The HA1 sequence used in this study has been successfully expressed in insect cells and determined by Western Blotting assay using specific monoclonal antibody in our lab (11). Although protein expression in the COS-7 and BHK-21 cells was not detectable using immunoassays, we determined heamagglutinin transcription in both cell lines using RT-PCR. In the following study we are going to evaluate immunogenicity of this DNA construct in combination with recombinant heamagglutinin prepared in insect cells and suitable adjuvant in animal model.

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