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Original Article

Construction of a Recombinant Bacmid DNA to Express Influenza Virus Matrix Protein1 (M1) in Insect Cell Line

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Abstract

Background and Aims: Virus-like particles (VLPs) have been suggested to be a promising recombinant vaccine approach. Several studies have reported that the influenza VLPs produced in insect cells is an effective vaccine candidate. Due to crucial role of matrix M1 protein in assembly and budding of Influenza particles, in all VLPs structures, M1 protein have been considered as a main component.

Methods: M1 open reading frame (759 bp) from human influenza virus A /New Caledonia 20/1999/ (H1N1) was amplified by RT-PCR. The amplicone was cloned into pFastBac1 donor plasmid through KpnI/XhoI sites. After verification of clone by restriction analysis, it was subjected to automated sequencing. The M1 recombinant bacmid was subsequently generated and verified by PCR using M1 specific and M13 universal primers.

Results: results showed that a recombinant baculovirus containing correct and inframe sequence of Influenza M1 gene under control of polyhedrin promoter has been constructed.

Conclusion: The above-mentioned M1 recombinant baculovirus can be used with other individual recombinant baculoviruses expressing HA and NA genes to produce influenza VLPs in insect cell line.

Keywords: M1 protein; Influenza; Virus-like particles (VLPs)

Introduction

Influenza A viruses, members of Ortomyxoviridae family, are able to infect a large variety of animals as well as humans. Influenza A viruses with segmented-negative strand genome are classified based on the antigenic properties of their two distinct surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA) which are located on the outer host-cell-derived envelope. The Matrix protein (M2), a nonglycosylated integral membrane protein presents in outer membrane that acts as an ionic channel. The Matrix protein (M1) which is lined within the outer envelope interacting with the ribonucleoprotein (RNP) and plays an essential role in virion assembly, budding and release (1, 2).

Studies have shown that mutations on M1 and M2 genes, both encoded from segment 7, lead to changes in the morphology of the wild-type virion. These observations indicate the crucial role of these two proteins in virion structure (3).

Moreover, research on Influenza virus-like particle (VLP) formation clearly demonstrates the significant role of the M1 protein beside of the other structural components in assembly, formation and budding process, although it is driven by a single expression of the M1 protein

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(4). It has been shown that even VLPs lacking either HA or NA could be formed and released from the cell expressing M1 from recombinant DNA or virus infected cell (5, 6).

The recombinant baculovirus and insect cell expression system provides high levels of recombinant protein that undergoes post-translational modification like glycosylation (7, 8). Therefore, application of such system allows large quantity production of a desirable protein, in native configuration as a vaccine.

In this study M1 gene was isolated and amplified from H1N1 influenza virus and then a clone of M1 recombinant bacmid was constructed. This construct can be used to produce VLPs of different influenza genotypes because M1 sequences have remained evolutionarily stable for all recorded human and avian influenza A viruses during the past decades (9). M1 recombinant bacmid also can be used to produce large scale of M1 protein, suitable for vaccine production.

Methods

RNA Extraction and cDNA synthesis

The human influenza virus A /New Caledonia 20/1999/ (H1N1) was propagated and harvested in MDCK cells according to the standard protocols (10). Viral RNA was extracted from 200 µl of harvested cell culture supernatant using a commercial RNX-PLUS^{TM} solution (Cinnagen-Iran). The viral RNA was eluted in 30 µl DEPC-treated water. The cDNA synthesis was performed using RevertAid First Strand cDNA Synthesis Kit (Fermantas-Lithuania) according to manufacturer's instructions. The primer Uni12 5'-AGCRAAAGCAGG-3', which is complem-entary to the conserved 3'end of all influenza A virus RNA segments was used for cDNA synthesis (11).

RT-PCR

A set of forward and reverse primers were designed by Gene Runner 3.05 (Hastings software, Inc) to amplify the complete M1 ORF of H1N1 Influenza virus (759bp). 5µl of cDNA was added to a total 20µl of PCR mixture containing 20pmol of each forward primer 5'AGCTCGAGATGAGTCTTCTAAC CGA 3' and reverse primer 5'ACTGGTACCT CAC-TTGAATCGTTGCA 3' (underlined nucleotides correspond to XhoI and KpnI sites respectively), 10 mM Tris-Cl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM dNTPs, High Fidelity PCR Enzyme Mix (Fermentas-Lithuania) and covered with 50 µl of mineral oil. Amplification reactions were performed in a thermocycler (Master Cycler, Eppendorf) under the following profile: 3 min at 95°C followed by 30 cycles at 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, with a final extension step at 72°C for 10 min. PCR products were analyzed on 1% (w/v) agarose gel electrophoresis stained with ethidium bromide upon preparation (12, 13).

Cloning of the M1 gene

The RT-PCR product was extracted and cleaned up from the agarose gel using DNA extraction kit (Bioneer-Korea) and subsequently cloned into the KpnI and XhoI sites of the pFastBac1 donor plasmid (Invitrogen-USA). The recombinant vector pFastBacM1 was confirmed using restriction enzyme analysis. Subsequently it was subjected to automatic one- directed sequencing using forward primer as described previously for M1 amplification.

Generation of recombinant bacmid

The recombinant donor plasmid, pFastBacM1, was transferred into the E.coli DH10Bac competent cells for site-specific transposition of the M1 DNA from the transposing vector to a bacmid DNA through lacZ gene disruption. The transformed cells were plated onto the LB agar containing kanamycin (50µg/ml), gentamicin (7µg/ml), tetracycline (10µg/ml), X-gal (100µg/ml) and isopropylthio-β-galactoside (IPTG, 40µg/ml) and incubated at 37ºC for 48h. The high-molecular-weight bacmid DNA was isolated from the overnight cultures by alkaline lysis purification according to the instructions supplied by the

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manufacturer (Invitrogen-USA). Successful transposition was verified by PCR analysis using either M13/pUC or M1-specific primers.

Results

Amplification and characterization of the M1 gene

The full length sequence of M1 gene (759bp) was amplified by RT-PCR using the designed specific primers (Fig 1). The fidelity of the M1 open reading frame (ORF) in pFastBacM1 was confirmed by sequencing. Analysis of sequencing was accomplished by Chromas software (version 1.45 - Australia).

Construction of the recombinant bacmid DNA

The M1 gene was cloned into pFastBac1 plasmid and verified by restriction endonuclease digestion in comparison with pFastBac1, as control (Fig 2). Following the transformation and plating of the cells on LB agar, the transposed colonies were visible as large white colonies among the blue ones harboring the unaltered bacmid. The selected white colonies were restreaked onto LB agar plate to ensure if they have true white phenotype.

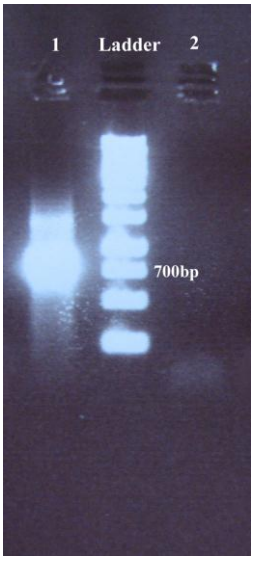

Fig. 1. Gel electrophoresis of M1 gene RT-PCR products, Lane 1: demonstrating thick bond of 759bp amplicone run along with molecular weight DNA marker Lane 2: negative control (blank).

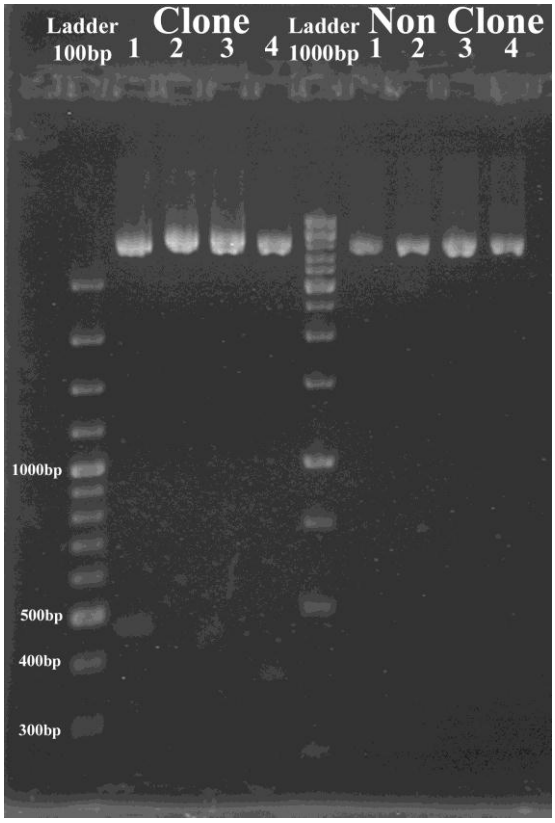

Fig. 2. Pattern of restriction enzymes digestion confirming new recombinant pFastBacM1. HindIII/XhoI double digestion on pFastBacM1(clone) revealed expected 304bp, 457bp and 4862bp fragments (lane1). Single digestion of KpnI and XhoI made the construct linear and released 5639bp bands (lane 2 and 3). BamHI digestion also generated expected 359bp, 5280bp fragments (lane 4). Whereas, all such digestions made pFastBac (nonclone) linear with 4775bp in length (lanes 1-4 on right panel). 100bp and 1Kb molecular weight DNA marker (Fermentas) applied as scale.

Since verification of the high-molecular weight recombinant bacmid DNA by digestion is not convenient, PCR was performed using M13/pUC and M1-specific primers to ensure proper transposition of the target gene in the recombinant bacmid. The bacmid DNA contains M13 forward and reverse priming sites flanking the mini-att Tn7 site within the LacZ α-complementation region. A panel of PCR reactions was done using M1 specific primers, M13/pUC primers, M1 gene specific forward and M13 reverse primers, and M1

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gene specific reverse and M13 forward primers for 759bp, 3059bp, 2074bp, 2725bp PCR products respectively. Amplification of the nonrecombinant bacmid using M13/pUC primers generated a 303bp band (Fig 3).

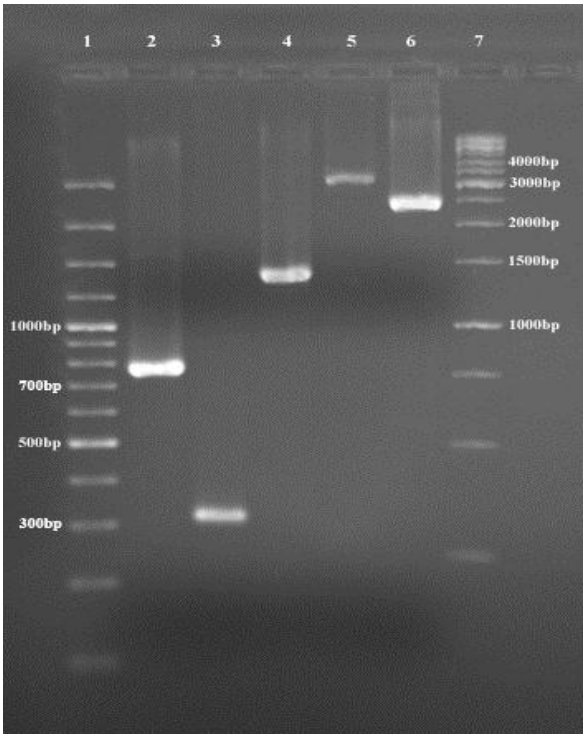

Fig. 3. A panel of PCR done to confirm M1 recombinant Bacmid

Lane 1, 100 bp molecular weight DNA marker (Fermentas).

Lane 2, PCR product using M1 specific primers on recombinant bacmid extracted from white colony (759bp).

Lane 3, PCR product using M13 specific primers (303bp) on nonrecombinant bacmid extracted from blue colony as a negative control.

Lane 4, PCR product using M1 forward and M13 reverse primers (~1400bp) on recombinant bacmid extracted from white colony.

Lane 5, PCR product using M13 specific primers (3059bp) on recombinant bacmid extracted from white colony.

Lane 6, PCR product using M13 forward and M1 reverse primers (2507bp) on recombinant bacmid extracted from white colonies.

Lane 7, 1kb molecular weight DNA marker (Fermentas).

Discussion

The expression of eukaryotic genes using baculovirus expression vectors takes advantages of their protein synthesis machinery and facilitates proper folding and post-translational modifications including glycosylation, acetylation, olygomerization and proteolysis. In addition, the insect cell cytoplasmic environment provides appropriate disulfide bridge assembly (14, 15). These features are important in the case of influenza A M1 protein, as its proper structure is critical for its function as a nexus that gather ribonucleoprotein complex and outer envelope-bound HA, NA, and M2 proteins together for packaging in host cell (5, 16). Production of Influenza VLP was demonstrated with different expression systems including recombinant DNA expression vectors (17), recombinant vaccinia viruses (16), and DNA plasmid transfection with T7 RNA polymerase-expressing vaccinia virus (5). However recombinant baculoviruses/ insect cell expression system yields high levels of recombinant proteins and more important, VLPs have been formed in such systems have been shown highly immunogenic both in neutralizing antibodies and cellular immune responses. Several research groups have reported the expression of the M1 Influenza in baculovirus-infected insect cells. Most of them planned to produce Influenza A virus like particles (VLPs) of different genotypes and apply them in vaccine research studies (18, 19, 20, 21).

Gomez-Puertas and colleagues studies have clearly demonstrated the significance of M1 protein in the assembly and budding process of influenza VLP formation (5, 22). Therefore, in the most influenza VLP studies, M1 protein has been considered as an essential component (18, 19, 20, 21). It is also demonstrated that VLPs lacking either HA or NA have been formed and released from cells expressing M1 through recombinant DNA or virus infection (16). In other word, even expression of M1 protein alone is sufficient to induce particle formation. In this work, a recombinant

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baculovirus containing correct and inframe sequence of Influenza M1 gene under control of polyhedrin promoter has been constructed. Owing to our ultimate goal to produce H1N1 influenza VLP, we chose the Bac-to-Bac expression system (Invitrogen life technologies) because apart from the above mentioned advantages, it is a valid and multi-purpose system for the production of the recombinant proteins.

In ongoing study, the M1 recombinant bacmid constructed here will transfect into the cultured Sf9 (Spodoptera frugiperda) insect cell line to produce recombinant baculovirus expressing M1 gene. Recombinant baculovirus expressing M1 gene can be used with other individual recombinant baculoviruses expressing HA and/or NA genes to produce influenza VLPs. Moreover, the M1 protein expressed in this system after purification is applicable in several vaccine research projects.

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