

Full Length Research Paper

Expression of recombinant Hepatitis C virus (HCV) Core, E1 and E2 proteins by the baculovirus expression vector system

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Hepatitis C virus (HCV) infection is definitely a global severe health problem. Neither a vaccine to prevent the infection nor an effective treatment for all HCV genotypes is available at the present time. The prophylactic HCV vaccine researches are recently focusing on eliciting antibody responses to three highly immunogenic structural proteins containing core protein (C) and envelope glycoproteins E1 and E2. The synthesis of the structural proteins-containing virus-like particles (VLPs) may provide us a useful tool to clarify the structural requirements for the assembly of HCV particles. HCV like particles also draw attention for their potential role in HCV vaccine development. In this study, HCV genotype-1a genomic RNA from the serum sample of a chronically-infected patient subjected to reverse transcription polymerase chain reaction (RT-PCR) and cloning into baculovirus pFastBacHTB vector. After vector's restriction analysis and sequencing for verification, the Bac-to-Bac system successfully generated the CE1E2 recombinant bacmid, which then transfected into Sf9 insect cells to produce the recombinant baculovirus. Finally, analysis through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting demonstrated the expression of recombinant CE1E2 protein. The described system represents an efficient means of simultaneous HCV structural proteins expression which may potentially be used for vaccine development and/or diagnostic purposes.

Key words: Hepatitis C virus (HCV), core, E1, E2, expression, insect cells.

INTRODUCTION

Hepatitis C virus (HCV) infection is a severe health problem that afflicts 180 million people worldwide. It is estimated that 3 to 4 million people are infected with this virus annually (Brooks et al., 2007; Lemon et al., 2007). HCV causes acute and chronic hepatitis which can result in permanent liver damage and hepatocellular carcinoma. At the moment, there is no vaccine available for

prevention of HCV infection because of the high degree of strain variation (Brooks et al., 2007; Torresi et al., 2011).

HCV is an enveloped, positive-sense, single-stranded RNA virus of the *Hepacivirus* genus of the Flaviviridae family. The HCV genome is approximately 9.6 kb in length that consists of an open reading frame encoding a polyprotein of about 3000 amino acids, and UTRs located at the 5' and 3' termini. The HCV polyprotein cleaves to structural and non-structural proteins using cellular and viral protease. The HCV structural proteins contain the nucleocapsid or core protein (C) and the two envelope

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glycoproteins, E1 and E2 (Aghasadeghi et al., 2006; Brooks et al., 2007; Lemon et al., 2007; Roohvand et al., 2007). A tendency to aggregation is a feature of the HCV glycoproteins, and possibly plays a crucial role in virus pathogeny. E1E2 heterodimers also enable the virus to bind to the cell and then enter it. Since the efficiency of virus replication in suitable cell cultures is low and there are no sufficient cell cultures and animal models, it is essential to search for alternative procedures to study HCV. An alternative model of the virion is provided by virus-like particles (VLPs), which are developed in insect or mammalian cells expressing the HCV structural genes (Belzhelarskaya et al., 2010). The baculovirus expression vector system (BEVS) has two characteristics which make it proper for HCV protein expression. First, eukaryotic insect cells are known to perform several co- or post-translational modifications, such as fatty acid acetylation and glycosylation, just like mammalian cells. Second, unlike many mammalian cell expression systems, the baculovirus expression system makes high-level production of heterologous proteins possible (Xiang et al., 2002; Zhao et al., 2004). Using BEVS, production of self-assembled, non replicating particles that resemble intact virions for many viruses becomes possible (O'Reilly, 1997; Baumert et al., 1998). The formation of HCV virus like particles (VLPs) in insect cells has a vital role in studying viral assembly and virus cells interactions and finally developing the HCV vaccine (Baumert et al., 1999; Belzhelarskaya et al., 2010; Beljelarskaya, 2011).

In this study, we constructed recombinant pFastBacHTB plasmid containing the cDNA of the HCV structural proteins in order to produce recombinant bacmid in *Escherichia coli* DH10Bac cells, which then transfected into Sf9 cells to produce core-E1-E2-expressing recombinant baculovirus. The potentially assembled VLP may be used for immunostimulatory and diagnostic aims.

MATERIALS AND METHODS

RNA extraction and cDNA synthesis

HCV RNA was extracted from serum of a chronically infected patient with HCV genotype-1a using High Pure Viral Nucleic Acid kit according to the manufacturer's instructions (Roche, Germany). cDNA was synthesized from total viral RNA using Random Hexamer Primer (Fermentas, Lithuania).

Polymerase chain reaction (PCR) amplification

To amplify the CE1E2 genes of HCV (2256 nt), 5 µl cDNA was added to 25 µl of PCR mix containing 10 pmol of each forward 5'-cggatccatgagcacaatcctaaac-3' and reverse 5'-attctagattactctccaatgctgcttc-3' primers (the underlined nucleotides demonstrate *Bam*HI and *Xba*I digestion sites, respectively), 1X PCR reaction buffer, 2 mM of MgCl₂, 200 µM of deoxynucleotide triphosphates (dNTPs) and 0.5 U of high fidelity PCR enzyme mix (Fermentas, Lithuania) in a Primus 25 thermocycler (Peqlab, Germany). The PCR condition included a primary denaturation of 3

min at 95°C, followed by 31 cycles of 1 min at 94°C, 1 min at 57°C and 2½ min at 72°C and a final extension phase at 72°C for 10 min. To analyze the PCR product, agarose gel electrophoresis was applied.

Cloning of the CE1E2 genes

The CE1E2 segment was gel-extracted using the high pure PCR product purification kit (Roche, Germany) and cloned through the *Bam*HI and *Xba*I sites into the pFastBacHTB donor vector (Invitrogen, USA), creating the pBaCE1E2 plasmid. The cloning was evaluated with restriction analysis by the same restriction enzymes, and the fidelity of the cloning was verified by sequencing (Eurofins, Germany).

Construction of recombinant bacmid

E. coli strain DH10Bac was transformed with the pBaCE1E2 recombinant donor plasmid. To perform site-specific transposition of the CE1E2 fragment from the donor plasmid to the bacmid DNA which already available in DH10Bac *E. coli* cells, the transformed DH10Bac cells were transferred to the Luria-Bertani broth (LB) agar plates containing gentamicin (7 µg/ml), kanamycin (50 µg/ml), tetracycline (10 µg/ml), X-gal (100 µg/ml) and isopropylthio-β-galactoside (IPTG, 40 µg/ml) and incubated at 37°C for 48 h. Afterward, the largest white colonies were re-streaked on the new LB agar plates containing the mentioned reagents to reconfirm their white phenotype. Finally, the confirmed white colonies were overnight cultivated in LB broth medium containing the mentioned antibiotics and subjected to bacmid extraction using plasmid extraction mini kit according to the manufacturer's instructions (Bioneer, Korea). In order to confirm the extracted recombinant bacmids, PCR was performed using universal M13 forward and reverse primers as instructed by the manufacturer (Invitrogen, USA).

Cell culture and transfection

Sf9 cells (Invitrogen, USA) were grown in Sf-900 III serum free medium (Invitrogen, USA). Afterward, they were transfected by the confirmed recombinant bacmid using Cellfectin II reagent as instructed by the manufacturer (Invitrogen, USA).

Viral titer determination

Following two consecutive rounds of viral particle amplification (P1 and P2), the amplified viral stock was subjected to viral titer determination by TCID₅₀ method as instructed elsewhere (O'Reilly et al., 1992; Masroori et al., 2010).

Expression assay

Sf9 cells were infected at multiplicity of infection (MOI) of 10 (pfu/cell), and 72 h post infection, insect cell lysates were evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analyses. The amount of expressed proteins was quantified by densitometric analysis of SDS-PAGE gel (LabWorks 4.0 software, UVP). For western blotting, the cell lysates were separated by SDS-PAGE and then transferred to the nitrocellulose membranes (Sambrook and Russell, 2001). Afterwards, the membranes were blocked with 3% skimmed milk + 0.1% Tween-20 followed by washing with phosphate-buffered saline (PBS). For detection of the CE1E2, one membrane was

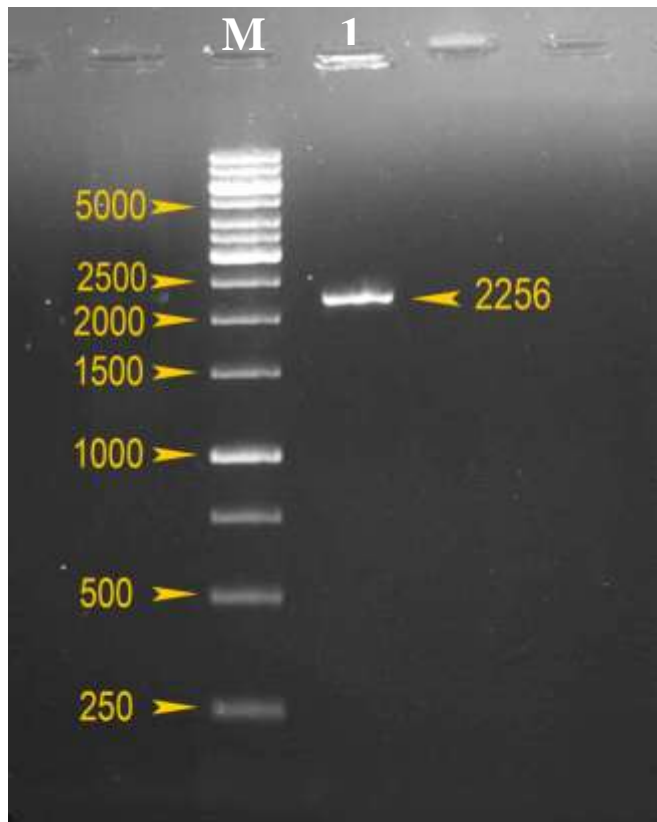


Figure 1. PCR amplification of CE1E2 fragment. Lane M, 1Kb DNA ladder; Lane 1, The PCR product of CE1E2 fragment (2256 bp).

treated with pooled anti-HCV positive serum of genotype-1a infected patients, and the other membrane was incubated with mouse monoclonal anti-core antibody (1:1000 dilution) (ABR-Affinity BioReagents, Inc) for 1 h. The membranes were then washed four times with PBS containing 0.1% Tween-20 and incubated for 1 h with horseradish peroxidase (HRP)-conjugated anti-human antibody (1:1000 dilution) (Sigma-Aldrich, Germany), and HRP-coupled anti-mouse secondary antibody (1:10000 dilution) (Sigma-Aldrich, Germany), respectively. Finally, the membranes were washed four times with PBS containing 0.1% Tween-20 and developed by chromogenic reagent, diaminobenzidine (DAB) (Roche, Germany).

RESULTS

CE1E2 amplification and cloning

The CE1E2 cDNA was obtained by reverse transcription, and amplified through PCR by specific primers (Figure 1). The amplified fragment was cloned into pFastBacHTB plasmid and the fidelity of the cloning was confirmed by restriction digestion (Figure 2) and sequencing.

Construction of the recombinant bacmid DNA

Following the transformation of DH10Bac *E. coli* cells



Figure 2. The product of restriction digestion. Lane M, 1Kb DNA ladder; Lane 1, pFastBacHTB donor plasmid (4856 bp), CE1E2 fragment (2256 bp).

with pBaCE1E2, blue/white screening was performed as mentioned. After confirmation of the white colonies by re-streaking, the extracted recombinant bacmids were verified by PCR as a 4686 bp amplified product (Figure 3). Also, for verification of the non-recombinant bacmids as negative control, we indicated the amplified 300 bp fragment (Figure 3).

Expression assay of the CE1E2 genes

For detection of CE1E2 protein, Sf9 cells which were infected with recombinant viruses at MOI of 10 were lysed 3 days post-infection. Densitometric analysis of SDS-PAGE gel determined the quantity of the recombinant protein as 20% of the total cell protein (data not shown). The western blotting using pooled HCV-antiserum revealed the different bands of about 36, 68 and 21 KD, indicating the expression of E1, E2 and core proteins, respectively (Figure 4A). Furthermore, western blotting using anti-core antibody confirmed the expression of the core protein (Figure 4B).

DISCUSSION

Although significant findings have been achieved in studying the HCV genome and virus proteins since the virus RNA was cloned in 1989, the structural features of

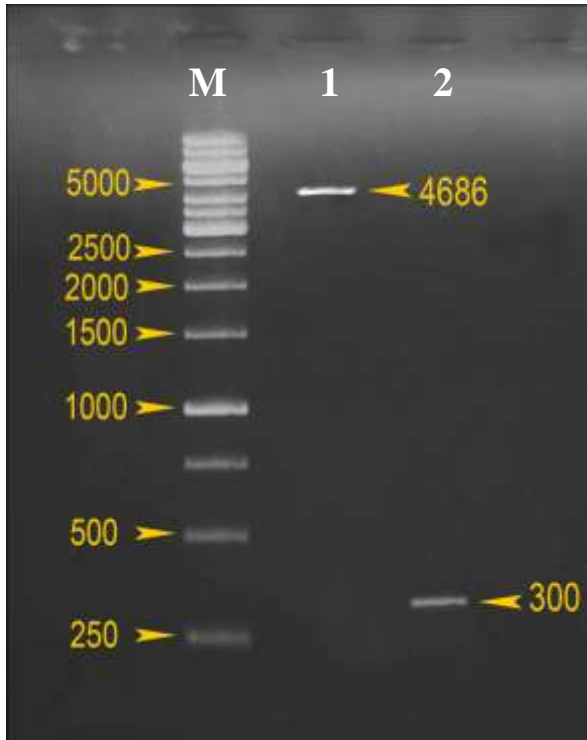


Figure 3. Confirmation of the recombinant bacmid. Lane M, 1Kb DNA ladder; Lane 1, recombinant bacmid; Lane 2, non recombinant bacmi

the virion are yet to be understood (Mihailova et al., 2006; Mancini et al., 2009). Indeed, small animals are not included in the limited host range of HCV, so the laboratory models cannot be used for virus proliferation. Since the efficiency of virus replication in proper cell cultures is low, and there are no simply available animal models, it is necessary to look for suitable substitutes to study HCV (Baumert et al., 1999; Elmowalid et al., 2007). The use of VLPs, which are produced in insect and mammalian cells, expressing the HCV genes for the structural proteins, can be an alternative model (Belzhelarskaya et al., 2010; Beljelarskaya, 2011). VLPs are self-assembled, non-replicating, genome-free particles that are the same in size and shape as the intact virions, and are useful tools for immunological studies (Belzhelarskaya et al., 2010; Beljelarskaya, 2011). These particles have been taken from both envelope-lacking viruses, such as the Poliovirus, Papillomavirus and Rotavirus, and also enveloped Retroviruses (Soleimanjahi and Fotouhi, 2009; Beljelarskaya, 2011). These findings were achieved from the development of highly efficient heterologous gene expression systems, as those based on recombinant baculoviruses (Chun and Chiang, 2010). VLPs, which are comprised of the structural proteins, can be utilized as a virion model to analyze the entry of the virus into the cell and the virus morphogenesis, and also a useful tool for immunological

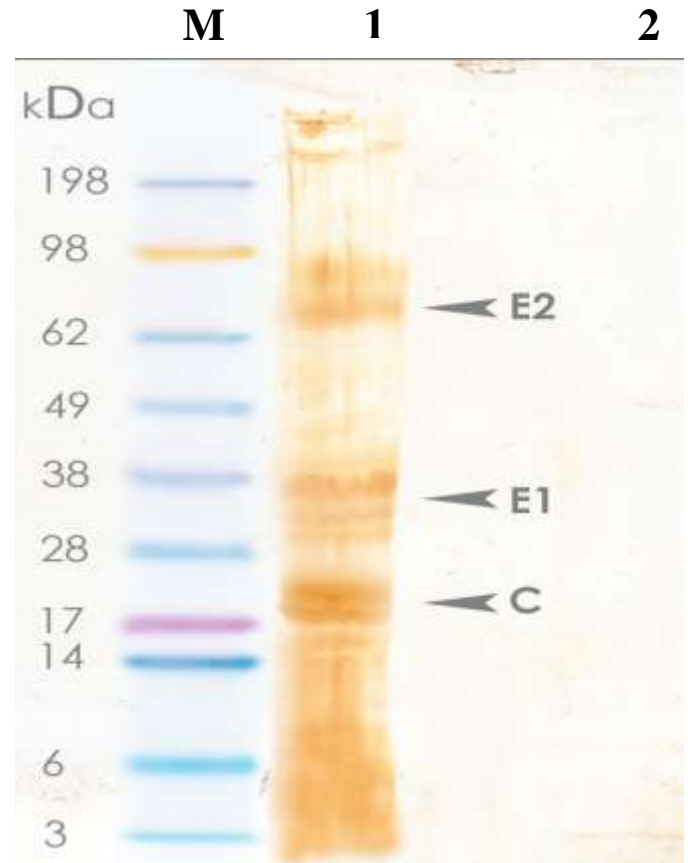


Figure 4A. E1, E2 and core proteins expressed in insect cells. Lane M, Pre-stained protein standard; Lane 1, the position of expressed E1, E2 and core proteins is indicated by arrows; Lane 2, Mock transfected insect cells as negative control.

studies (Grgacic and Anderson, 2006; Buonaguro et al., 2010). Insect cells which are infected with a recombinant baculovirus give a proper model for studying the assembly of virus particles and for developing the prototype vaccines on the basis of VLPs and recombinant antigens (Anderson et al., 1995). Accordingly, we planned to analyze the production of hepatitis C structural proteins by means of Bac-to-Bac baculovirus expression system in order to obtain potential VLPs (Belzhelarskaya et al., 2010). To produce CE1E2 recombinant protein which is able to form VLP, the HCV genotype-1a positive serum was used to provide target cDNA; and based on Bac-to-Bac mechanism, the protein was expressed and analyzed by SDS-PAGE and western blotting methods. The presence of polyhedrin promoter in the baculovirus expression system can lead to high level of target gene expression in transcription phase and finally results in increased production of the target proteins (Chun and Chiang, 2010). Additionally, the ability of post-translational modifications such as glycosylation, phosphorylation and oligomerization make the recombinant proteins have similar structure and function

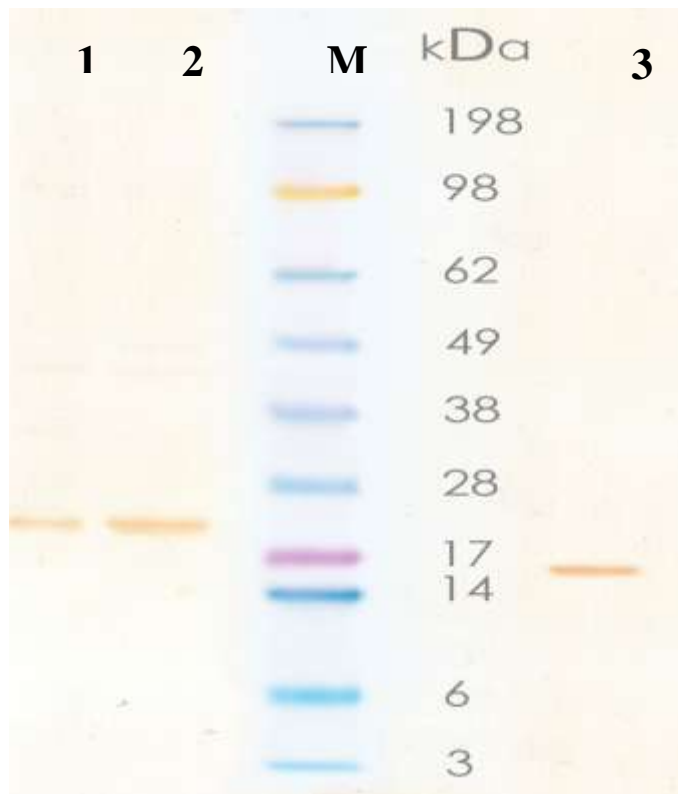


Figure 4B. Expression of the core protein. Lane 1 and 2, The core protein expressed; Lane M, pre-stained protein standard; Lane 3, core protein expressed in bacterial plasmid as positive control.

as the intact proteins (Soleimanjahi and Fotouhi, 2009; Belzhelarskaya et al., 2010; Beljelarskaya, 2011). Accordingly, due to signal peptides among core, E1 and E2 proteins, they have been processed by cell signal peptidases, and also glycosylated which resulted in a heterogeneous SDS-PAGE band pattern in this study that was similar to the several previous studies (Beljelarskaya, 2011). Despite a previous study based on pBlueBac 4.5 vector for genotype-1a VLP formation (Xiang et al., 2002), to our knowledge, this is the first report of CE1E2-based VLP formation of HCV genotype-1a by means of Bac-to-Bac mechanism.

In conclusion, in order to obtain VLPs and applying them together with new adjuvants for analyzing the immunization in the future, we expressed HCV structural proteins through baculovirus expression system. Also, the potential VLPs might be used as heterologous vectors for diagnosis and therapy purposes.

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