

# Immunogenicity of HGV NS5 protein expressed from Sf9 insect cells

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## INTRODUCTION

Although reliable assays for the detection of hepatitis C virus and E virus became available, still 10%-20% hepatitis are not caused by hepatitis A-E virus<sup>[1-3]</sup>. In 1996, two research groups isolated this agent independently and almost simultaneously and named hepatitis G virus and GB virus C, respectively<sup>[4-7]</sup>. The nucleotide and amino acid homologies between GBV-C and hepatitis G virus (HGV) were 85% and 95%<sup>[5-7]</sup>. Therefore, GBV-C and HGV were considered as two different isolates of the same virus, referred to as HGV in this paper. HGV is a single-strand, positive sense RNA virus with approximately 9.4kb in length, and classified as a member of *Flaviviridae*. HGV is mainly transmitted through transfusion and could be responsible for chronic liver infection. HGV RNA has been detected in the serum of intravenous drug users (IVDUs), volunteer and commercial blood donors, and patients with cryptogenic hepatitis<sup>[8-10]</sup>. Until now, RT-PCR is the most commonly used method for the diagnosis of HGV infection. It is necessary to develop a more convenient antibody detection assay. The baculovirus expression system is of a strong polyhedrin promoter<sup>[11]</sup>, and can carry out many types of postranslation modification for a variety of proteins. Most of the expressed proteins were usually shown to be antigenic, immunological, and functionally similar to their authentic counterparts<sup>[12-16]</sup>. In this study, we used the baculovirus expression system to express HGV NS5

protein in Sf9 cells, and studied its immunogenicity.

## MATERIALS AND METHODS

### Materials

HGV positive sera were collected from HGV RNA positive hemodialyzed patients. The plasmid pFastBac HTa, *E. coli* DH10 Bac cell, *Spodoptera frugiperda* (Sf9) cell and recombinant plasmid HGV Iwh6 were prepared previously in this laboratory<sup>[17]</sup>. The pPROEX HTa, Lipofectin and Grace's medium were purchased from GIBCO/BRL; and expand<sup>TM</sup> Long Template PCR System was purchased from Boehringer Mannheim Company. PCR primers were designed according to HGV-Iwh6 and synthesized by Sangon Biotechnology Company. Two restriction enzyme sites *Bam* H I and *Kpn* I were added to the 5' end of sense and antisense primers separately. The primer sequences are sense: 5'-GCG GAT CCC TAT CGG CTG CTG TAG CTA AG-3'; antisense: 5'-GCG GTA CCT TAT TGA GCG GCC CTC TTA GC-3'.

### Amplification and sequence analysis of HGV NS5 fragment

HGV NS5 fragment was amplified using HGV-Iwh6 clone as the template (PCR condition: predenature 94°C 2 min, followed by 94°C 30 s, 60°C 1 min, 68°C 2 min, 35 cycles, and extension 10 min before the ending of the reaction). The amplified fragments and pPROEX HTa were digested with *Bam* H I and *Kpn* I. Fragment and vector were recovered respectively and ligated by T4 DNA ligase to obtain recombinant plasmid pHTNS5. Sequence analysis was carried out using ABI PRISM 377 DNA sequencer (PE Company) with M13/pUC primer.

### Cloning into transposing vector pFastBac HTa

pHTNS5 and transposing vector pFastBac HTa were digested with *Bam* H I and *Kpn* I, and were ligated by T4 DNA ligase. The ligation mixture was transformed into DH5 $\alpha$  competent cell, the positive colonies were chosen on selecting agar plate (ampicillin 100  $\mu$ g/mL) and identified with endonuclease digestion to obtain the recombinant plasmid pFHTNS5.

### Transposon between pFHTNS5 and bacmid

Plasmid pFHTNS5 was transformed into DH10Bac competent cells containing bacmid with a mini-att Tn7 site and helper plasmid. Following hot-shock at 42°C for 45 s, the transformation mixture was placed in a shaking incubator at 37°C for 4 h. Recombinant

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bacmid was selected on selecting plate agar containing kanamycin 50 µg/mL, gentamicin 7 µg/mL, tetracycline 10 µg/mL, X-gal 200 µg/mL, and IPTG 40 µg/mL after 24 h-48 h incubation at 37°C.

#### Transfection of Sf9 cells

Recombinant bacmid was extracted according to the procedure of Bac-to-Bac system. For transfection, Sf9 insect cells were grown to 60%-70% confluence. The recombinant bacmid DNA 2 µg was transfected into insect cells Sf9 with Lipofectin. After 5 d-6 d incubation at 27°C until the morphology of the cells had obvious changes, Sf9 cells and viral supernatant were harvested respectively.

#### Expression of recombinant protein in insect cells and SDS-PAGE, Western-blot analysis

Twenty µL viral supernatant harvested from the transfected cells was used to infect fresh insect cells. After 5 d-6 d incubation at 27°C, the cells were harvested for protein expression analysis. The cells were washed twice with PBS and analyzed by SDS-PAGE according to the standard procedure. Western-blot was performed using HGV RNA positive sera (1:40 dilution).

## RESULTS

#### Amplification of HGV NS5 fragment and sequence analysis

PCR product was analyzed by agarose gel electrophoresis and the length was the same as expected (Figure 1). Sequence analysis showed that the HGV NS5 fragment was cloned into the vector with correct orientation (data not shown).

#### Construction of recombinant transposing plasmid pFHTNS5

Figure 2 shows the construction of recombinant transposing plasmid pFHTNS5. Figure 1 shows the analysis of recombinant plasmid on agarose gel by restriction endonuclease digestion which verified that target fragment was correctly cloned into the transposing vector. The results demonstrated a successful construction of recombinant transposing plasmid pFHTNS5.

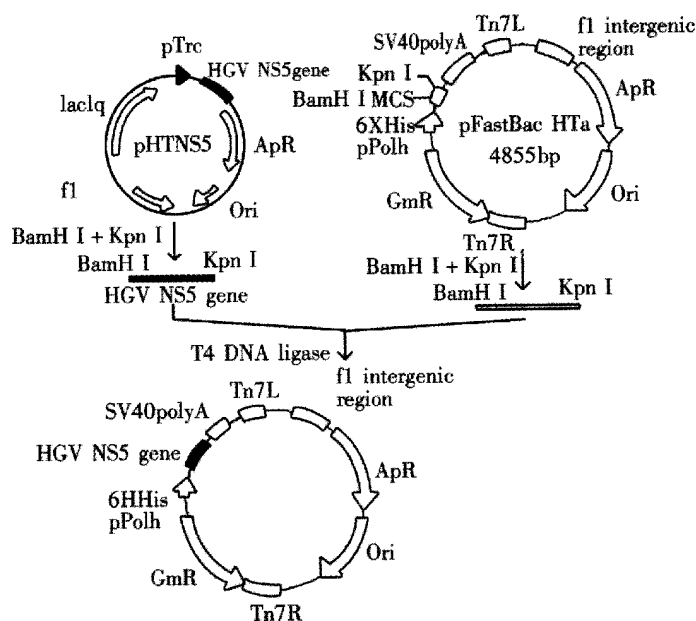


**Figure 1** Analysis of recombinant plasmid by restriction endonuclease digestion.

1.  $\lambda$ DNA/*Eco*RI + *Hind*III; 2. PCR product; 3. pHTNS5/*Bam*HI + *Kpn*I; 4. pFHTNS5/*Bam*HI + *Kpn*I.

#### Screening of recombinant bacmid

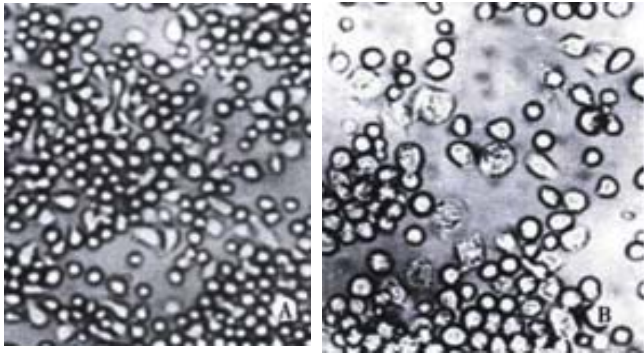
After transforming competent cell DH10Bac with transposing plasmid pFHTNS, the recombinant bacmid was screened by colour selection. White clones (*lacZ*<sup>-</sup>) were selected as positive recombinant bacmid in a background of blue colonies (*lacZ*<sup>+</sup>). The recombinant bacmid was extracted according to the procedures described in the manual of Bac-to-Bac system.



**Figure 2** Construction of recombinant plasmid pFHTNS5.

**Morphology of transfected or infected Sf9 cells**

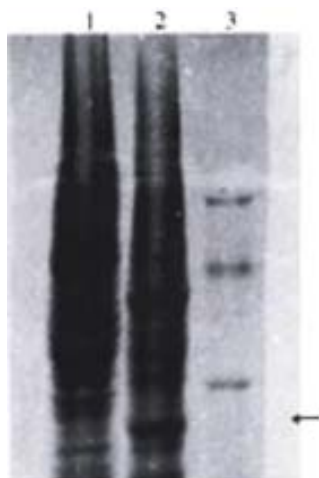
The morphology of Sf9 cells changed gradually after transfection or infection. The cells became big and round obviously at 4 d-5 d after transfection or infection. Cytopathic effects (CPE) were seen whereas no pathological effects were observed in normal cells (Figure 3).



**Figure 3** Morphology of uninfected, transfected and infected Sf9 cells (100×). A: Uninfected cells; B: Transfected and infected cells

**SDS-PAGE and Western blot analysis of the recombinant protein**

Transfected or infected Sf9 cells were harvested and analyzed on 12.5% polyacrylamide gels. Figure 4 shows the result of expressed target HGV NS5 protein with a molecular weight of  $M_r$  41 500. Scanning results indicated that the recombinant protein amounted to 11.7% of the total proteins. Western blot results implied that the recombinant protein could react with HGV RNA positive sera (Figure 5).



**Figure 4** SDS-PAGE analysis of expressed HGV NS5 protein. 1. Uninfected sf9 cells; 2. sf9 cells infected with recombinant viruses; 3. Protein relative molecular mass standards. Arrow indicates the position of recombinant protein.



**Figure 5** Western-blot analysis of recombinant protein HGV NS5. 1. Uninfected sf9 cells; 2. sf9 cells infected with recombinant viruses; 3. Protein relative molecular mass standards.

**DISCUSSION**

Although easy and reliable assays for the clinical diagnosis of HBV and HCV infection have been established<sup>[18-26]</sup>, there still existed 10%-20% parenterally and community acquired hepatitis cases of unknown cause<sup>[4,5,7]</sup>. Transmission and molecular biology of these viruses have been studied thoroughly<sup>[27-34]</sup>. Clinical studies suggest that some of these may be of viral origin. HGV is a potential aetiological agent for viral hepatitis. As a member of Flaviviridae, HGV is a single-stranded RNA virus with a genome of 9 400 bp in length which includes 5' non-coding region, structural gene region C, E1, E2, non-structural gene region NS2, NS3, NS4, NS5a, NS5b and 3' non-coding region. The genome contains a single open reading frame (ORF) which encodes a 2 900 amino acid polyprotein precursor. Many researches have been carried out since the discovery of HGV, the studies of its antigenicity is one of them<sup>[17,35-39]</sup>. HGV NS5B protein functions as RNA-dependent RNA polymerase]. In addition, Pilot-Matias *et al*<sup>[40]</sup> also found that C26, C27, C28 (2047-2376 aa) of HGV NS5 gene had potential antigen epitopes. Wang *et al*<sup>[41]</sup> reported that two linear epitopes (P22, P6) might exist in HGV NS5 gene. The obtained HGV NS5 recombinant protein will provide important materials for studying its structure and function.

The Bac-to-Bac system was established by Luckow<sup>[11]</sup> in 1993, and a variety of proteins have been expressed with the control of a strong polyhedrin promoter since then. It is based on site-specific transposition (transposon Tn7) of an

expression cassette into baculovirus shuttle vector (bacmid) propagated in *Escherichia coli*. After selection of blue-white clones, recombinant bacmid DNA was extracted for the transfection of Sf9 cells. Insect cells can identify and run many modifications of post-translation and make the expressed protein close to natural protein.

In this study, HGV NS5 gene fragment amplified by PCR was confirmed by restriction enzyme and sequence analysis, and cloned into baculovirus transposing vector pFastBacHTa. Recombinant bacmid was obtained with site-specific transposition, Sf9 cell was transfected with recombinant bacmid or infected with viral supernatant. On the polyacrylamide gel, an expected protein band was seen at  $M_r$  41 500. Western blot found that HGV NS5 recombinant protein could react strongly with HGV RNA positive sera, which implied that recombinant HGV NS5 protein could be used as antigen to detect HGV infection.

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