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Partial reconstitution of hepatitis C virus RNA polymerization by heterologous expression of NS5B polymerase and template RNA in bacterial cell

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Abstract

The hepatitis C virus (HCV) is a major etiological agent causing chronic hepatitis in humans. Since the virus does not grow in a cell culture, the direct measurement of viral replication is impossible. Therefore, the current study presents a surrogate model system using a viral polymerase and RNA template. A plasmid expressing the HCV NS5B polymerase was maintained with a plasmid containing a reporter gene in an *Escherichia coli* cell. The reporter construct contained the HCV 5′ untranslated region (UTR) followed by a luciferase gene with a specific orientation so that a minus-sense transcript containing the luciferase fused to the 5′ UTR was produced after the initial transcription. When the HCV NS5B polymerase was expressed in the same cell, the primary transcript was recognized by the polymerase due to the presence of the minus-sense 5′ UTR, and a secondary transcript containing a plus-sense luciferase gene was produced. Thus, a simple luciferase assay was able to measure the HCV NS5B polymerase activity. The production of minus- and plus-sense transcripts was confirmed by an RT-PCR, while the production of HCV NS5B and expression of the reporter luciferase in the bacterial cell were confirmed by immunofluorescence microscopy. The polymerization occurred in the absence of any other viral/host factors. Accordingly, this would appear to be the first study to demonstrate that the heterologous expression of an animal viral RNA polymerase and its template in a bacterial cell can partially reconstitute the polymerization reaction.

Keywords: Hepatitis C virus (HCV); NS5B polymerase; RNA replication; Bacterial cell

1. Introduction

The hepatitis C virus (HCV) causes chronic hepatitis and can lead to liver cirrhosis and hepatocellular carcinoma (Moradpour and Blum, 2004), with more than 170 million people currently infected worldwide. The virus belongs to the *Hepacivirus* genus of the flavivirus family (Moradpour and Blum, 2004), and contains a plus-sense single strand RNA genome of approximately 9.5 kilo bases (Rosenberg, 2001). Its untranslated regions (UTRs) have extensive secondary structures at the 5′ and 3′ termini (Rosenberg, 2001), where the 5′ UTR includes an internal ribosome entry site (IRES) for translation, while the 3′ UTR consists of a conserved X tail sequence of 98 nucleotides, poly U/UC region, and a variable region. The 3′ UTR has already been shown to serve as a template for recognition and nucleotide incorporation by NS5B polymerase in vitro, plus the 3′-terminus of the coding region has been shown to play a role in replication in cis (Lee et al., 2004). The virus also encodes a polyprotein later cleaved into at least nine individual proteins, including the NS5B RNA-dependent RNA polymerase (Rosenberg, 2001). The NS5B polymerase is a 65 kDa protein responsible for RNA replication (Behrens et al., 1996), as it recognizes the specific RNA sequence in the 3′ UTR of the genomic RNA and copies it to produce minus-sense RNA (Lohmann et al., 1997). Meanwhile, the initiation of polymerization has been reported to occur in a de novo fashion (Sun

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et al., 2000; Kolykhalov et al., 2000; Zhong et al., 2000; Luo et al., 2000), where the 3′ UTR region of the minus-sense strand (originally the 5′ UTR in the plus-sense strand) is then recognized by the polymerase and more plus-sense RNA is produced. Thus, the specific secondary structure formed in the 3′ UTR of minus-sense RNA is also critical for RNA replication. These RNA templates are then used for translation for the further production of viral proteins, and as genomic RNA packaged into progeny viral particles. Other viral factors, such as NS3 helicase, NS4A, and NS5A, have also been reported to be involved in the polymerization reaction (Ishido et al., 1998), the replication complex shown to be inserted in a membranous structure or lipid raft (Gao et al., 2004), and other host factors, such as p68 (Goh et al., 2004), involved in HCV replication.

Since the virus does not grow in an in vitro cell culture system, direct measurement of the viral replication is impossible. However, there are two systems in which HCV RNA replication has been measured. The first is a subgenomic replicon system in which RNA containing genes encoding an antibiotic marker and HCV non-structural proteins is continuously replicated in an animal cell line (Lohmann et al., 1999). The replicon is maintained by selective pressure in the presence of G418. Plus, a more recently developed replicon system enables expression of the whole genomic RNA (Ikeda et al., 2002). Meanwhile, the second system uses purified recombinant NS5B and a template RNA in a test tube reaction. A variety of tagged forms as well as native NS5B have been used and reported to be active in this assay (Oh et al., 1999, 2000), and the template contains either the 3′ UTR or an oligoU-polyA synthetic construct (Lohmann et al., 1997; Liu et al., 2004). Assay methods using non-radioactive materials (Park et al., 2002) as well as radioactive isotopes have also been reported, where it was shown that NS5B alone could copy an RNA template containing the HCV UTR in a test tube, and no other viral/host factors were necessary. Accordingly, based on these facts, this study developed a system that enables HCV NS5B polymerase activity to be measured in a bacterial cell. Thus, the activity of a heterologously expressed viral polymerase in a template RNA is investigated in a bacterial cell.

2. Materials and methods

2.1. Plasmid constructs and bacterial strain

Two plasmids were constructed and maintained in *Escherichia coli* BL21(DE3). pSKNS5 expressed HCV NS5B in pBlueScriptSK. The gene encoding NS5B without a hydrophobic 21-amino acid C-terminus region was cloned between the *Eco*RI and *Not*I sites. pSKNS3/5 expressed both NS3 and NS5B in pBlueScriptSK. A Shine–Dalgarno sequence was inserted upstream of each gene to ensure the translation. The expression was induced with IPTG. pACLuc was the reporter plasmid based on pACYC184 containing the HCV 5′ UTR (entire 341 nucleotide 5′-terminus plus 76 nucleotides from 5′-terminus of core-coding region of HCV genomic RNA) and firefly luciferase in the minus-sense (Fig. 1). The gene encoding tetracycline resistance was partially deleted at the *Hin*dIII and *Bam*HI sites. There was a bacterial ribosome recognition site (Shine and Dalgarno sequence) between the UTR and luciferase gene in the minus-sense. As a control, the same plasmid was cleaved with *Bam*HI and *Sma*I (there are 2 *Sma*I sites, at 139 and 326 bases from 5′-terminus in the 5′ UTR) to generate a reporter with a partially deleted 5′ UTR. The *E. coli* cell was transformed with the two plasmids and maintained in the presence of both ampicillin and chloramphenicol.

2.2. Luciferase assay

A fresh culture was grown for 2 h and induced with IPTG (final concentration of 1 mM) for 2 h. The cells were collected after brief centrifugation, and resuspended in 100 μl of a lysis buffer (1 mg/ml lysozyme, 20% (w/v) sucrose, 30 mM Tris–Cl, pH 8.0, 1 mM EDTA) with vortexing. The mixture was kept on ice for 10 min and the supernatant recovered after

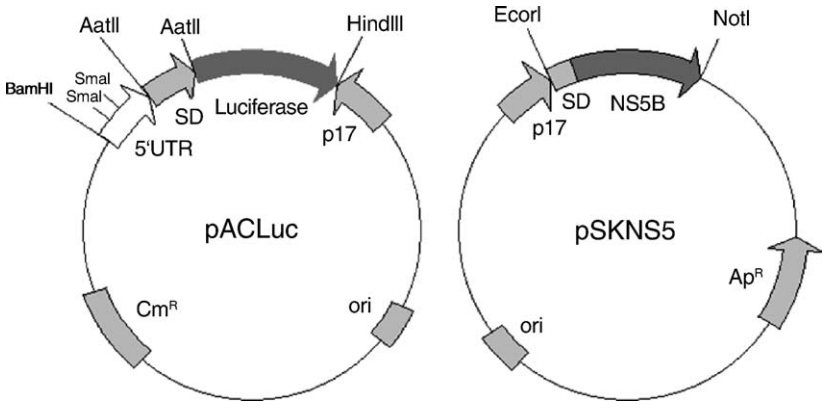

Fig. 1. Two plasmid constructs used in study. pACLuc is the reporter plasmid harboring firefly luciferase and the HCV 5′ UTR in the minus-sense, while pSKNS5 is the plasmid expressing the HCV NS5B polymerase. The two plasmids have replication origins belonging to different incompatibility groups so that they can coexist in the same bacterial cell. The restriction enzyme sites used are shown. SD, Shine–Dalgarno sequence; pT7, T7 promoter.