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Purification and Characterization of Recombinant Hepatitis C Virus Replicase

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Abstract The gene encoding the RNA-dependent RNA polymerase of the hepatitis C virus was cloned and expressed with a C-terminal hexahistidine tag. The protein was purified from *Escherichia coli* to near homogeneity and characterized *in vitro*. When the 21 amino acids from the C-terminus of the protein were deleted, an inclusion body was not formed and a better purification yield was achieved. However, the activity of the purified enzyme decreased compared to that of the full length protein. The purified enzyme did exhibit ribonucleotide-incorporation activity on an *in vitro* transcribed RNA containing the 3' end of the HCV genome. It also possessed ribonucleotide incorporation activity, to a lesser extent, on *in vitro* transcribed foreign RNA templates when RNA or DNA primers were present. The activity was higher with DNA primers than with RNA primers. Accordingly, this assay system will facilitate the screening of inhibitors for hepatitis C virus replication.

Key words: Hepatitis C virus (HCV), RNA-dependent RNA polymerase (replicase), cloning, purification, replicase assay

The hepatitis C virus (HCV) is a major etiological agent that causes chronic hepatitis in humans. The presence of this virus was first recognized in 1989 using molecular biological techniques [4]. Unlike hepatitis B, the development of a vaccine for this virus has been unsuccessful to date. HCV is a member of the *Flaviviridae* [8]. Like other viruses in this family, it contains a positive sense single-stranded RNA genome [5]. A long polymeric protein is produced from the RNA and cleaved to at least 10 separate proteins. Two nonstructural proteins, NS3 protease/helicase and NS5B replicase, are considered as the best targets for HCV therapy [2]. Here, we report an *in vitro* replicase assay system which is useful for screening inhibitors. NS5B includes the GDD motif which is highly conserved among the RNA-dependent RNA polymerase (RdRp) families [12]. Several groups have reported on the cloning of the gene encoding HCV replicase and the characterization of the enzyme [1, 3, 7, 10, 11, 13, 14]. It was reported that the protein expressed from either a baculovirus system or *Escherichia coli* showed comparable RNA-dependent RNA polymerase activity. However, one of the major obstacles is the insolubility of the protein when it is overexpressed from a bacterial system. In this study, the NS5B region was cloned in an expression vector that contained a C-terminal hexahistidine tag and then expressed in *E. coli*. Initially, it was attempted only to purify the full-length protein with an N-terminal tag, yet the resulting RNA-dependent RNA polymerase activity was minimal. Next, an attempt was made to purify the full-length protein with a C-terminal tag. This time, even though the purification yield was very low, it showed some RdRp activity. Finally, the protein was truncated at the C-terminus and purified in a C-terminal hexahistidine-tagged form; whereafter, it exhibited a very high purification yield and a decent RdRp activity.

The NS5B region of HCV was cloned as follows. The template DNA was pCITE-SX (a generous gift from Dr. Raffaele De Francesco [6]) which contained the cDNA encoding nonstructural proteins of HCV. The three primers used were: forward, 5' GCAGCAGGATCCTGCTCAAT-GTCTTAC 3'; reverse, 5' GATATAAAGTCCTCATCGG-TTGGGGAGGAGG 3' for cloning the gene encoding the full-length protein; and reverse, 5' GCGGGGTCGGGCA-CGAG 3' for the C-terminal 21 amino acid deleted protein. Each PCR product was ligated into the vector pET21-a(+) digested with the restriction enzymes *Bam*HI and *Hin*dIII to express C-terminally hexahistidine-tagged proteins, named pCP9 and pCP11, respectively. Each plasmid was then used to transform *E. coli* BL21 (DE3). The *E. coli* strains were induced with 1 mM IPTG for 3 h and crude extracts were obtained using a French Press. The crude extracts were then loaded on a DEAE sephacel column (Pharmacia, Sweden) and the flowthrough fraction was loaded on a Ni-NTA agarose resin (Qiagen, U.S.A.). The proteins bound to the resin were eluted with a buffer containing 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 150 mM imidazole.

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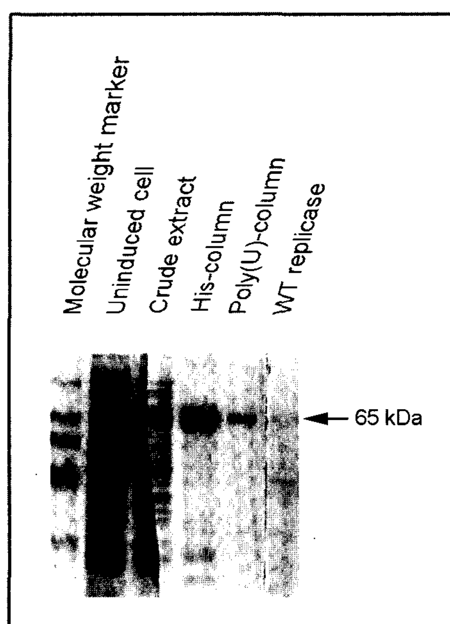

Fig. 1. The purification of recombinant HCV replicase.
Molecular weight markers are; 97.4, 66.2, 55.0, 42.7, 40.0, and 31.0 kDa from top to bottom. Uninduced cell, uninduced *E. coli* BL21 (DE3) harboring pCP11; Crude extract, crude extract of the same cell induced with IPTG; His-column, the eluted fraction from Ni-NTA column; Poly(U) column, the eluted fraction from poly(U) affinity column; WT replicase, the full-length protein expressed from pCP9 purified according to the same procedure as C-terminally-21-amino-acid-deleted protein.

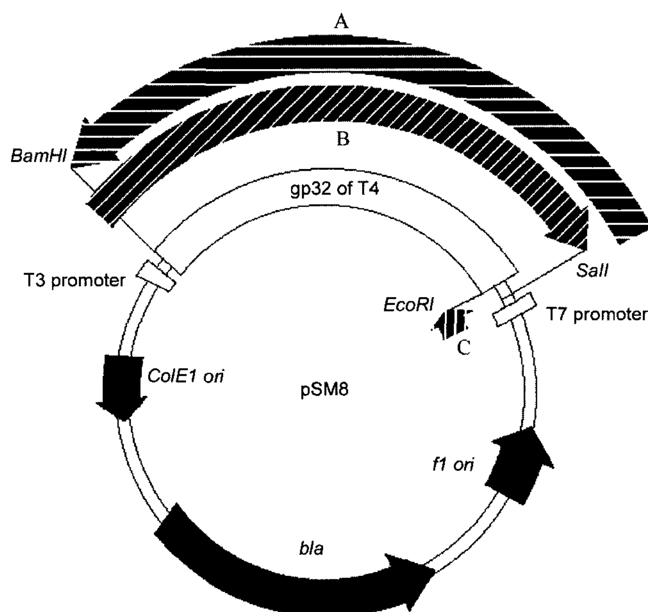

Fig. 2. Production of template and primer RNAs from pSM8 using *in vitro* transcription.
Each shaded arrow indicates the corresponding RNA template or primer produced. Shaded arrow A: RNA template for DNA primer; shaded arrow B: RNA template for RNA primer; shaded arrow C: RNA primer for RNA template.

The eluents were loaded on a poly(U) sepharose affinity column (Pharmacia, Sweden) and the affinity purified proteins were analyzed on an SDS-PAGE (Fig. 1). In the case of the full-length protein, the final protein yield was poor due to the formation of inclusion bodies. The C-terminally-21-amino-acid-deleted protein lost its hydrophobic 4-leucine motif and the purification yield was much higher. The SDS-PAGE analysis showed that the 65 kDa protein band was >99% pure as determined by a densitometer.

One of the templates for ribonucleotide incorporation assay was produced from pSM8 (Fig. 2). This was a plasmid vector pBlueScriptSK containing the gene encoding bacteriophage T4 gp32 (to be published). It contained the T7 promoter and was subjected to an *in vitro* transcription with the T7 RNA polymerase and 4 ribonucleotides after linearization with either the restriction enzyme *Sal*I when an RNA primer was used, or *Bam*HI when a DNA primer was used. The transcription mixture was then treated with DNase I and phenol:chloroform:isoamylalcohol. After ethanol precipitation, the RNA was used as the template for the HCV replicase assay. The deoxyribonucleotide primer (5' CTATTAAAGGTCATTCAAAAGG 3') which is complementary to the 3' end of the transcript, was synthesized from Genotech (Taejon, Korea). The ribonucleotide primer was produced by *in vitro* transcription. The same plasmid which produced the RNA template was used, however, it was transcribed from the opposite promoter using the T3 RNA polymerase after linearization with *Eco*RI. The T3 promoter-transcribed short RNA had a consecutive 30-basepair complementarity to the 3' end of the first RNA template. The other RNA template was produced from plasmid pBSK9286-9604/7, which was a generous gift from Dr. Ralf Bartenschlager [12]. It contained the 3' terminal nucleotide sequence of the HCV genome. The plasmid was subjected to a PCR using M13-20 primer (sense) and the following antisense primer: 5' ACATG-ATCTGCAGAGAGGCCAGTATCA 3'. The PCR product was then used for an *in vitro* transcription with the T7 RNA polymerase to generate a proper 3' end. The replicase assay mixture contained either a preannealed 20 μg RNA template and primer (either RNA or DNA) or RNA containing the HCV X-tail, 1 μg of the purified enzyme, a buffer with 20 mM Tris-Cl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 20U RNase inhibitor (Promega, U.S.A.), 25 μg/ml rifampicin, 5 μCi [^{35}S]-UTP, 0.5 mM CTP, 0.5 mM ATP, and 0.5 mM GTP. The reaction mixture was incubated at 30°C for 1 h. The reaction was stopped with heating at 75°C for 10 min and the mixture was then subjected to a sephadex G50 spun column to remove any unincorporated [^{35}S]-UTP. The reaction product was finally analyzed in a liquid scintillation counter for measuring its radioactivity.

From the purification procedure, a >99% pure replicase, 21 amino-acids deleted from the C-terminal was recovered

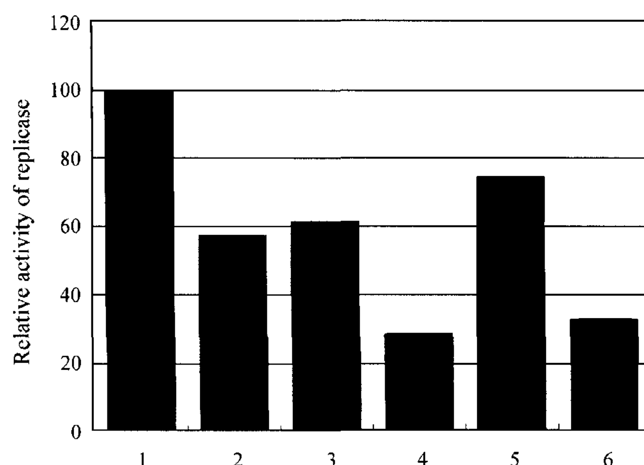

Fig. 3. RNA-dependent RNA polymerase activity of two types of purified proteins on various substrates.

100% activity is 27,500 cpm. 1. Activity of full-length protein on RNA transcribed from pBSK9286-9204/7 containing HCV X-tail sequence. 2. Activity of C-terminal truncated protein on RNA transcribed from pBSK9286-9204/7 containing HCV X-tail sequence. 3. Activity of full-length protein on RNA transcribed from pSM with DNA primer. 4. Activity of C-terminal truncated protein on RNA transcribed from pSM with DNA primer. 5. Activity of full-length protein on RNA transcribed from pSM with RNA primer. 6. Activity of C-terminal truncated protein on RNA transcribed from pSM with RNA primer.

(Fig. 1). The amount of the full-length protein recovered was less than 10% using the same procedure. The assay results of the replicase with various substrates are shown in Fig. 3. The highest activity was observed when the full-length replicase and HCV 3' X-tail RNA substrates were used. The foreign RNA substrates showed a 61-74% efficiency when compared to the authentic RNA. The C-terminal 21-amino-acid-deleted protein showed a 28-57% efficiency when compared to the full-length replicase. When the foreign RNA produced from pSM was used as the template in the absence of any primers, the enzyme activity was minimal (data not shown). The replicase seems to be able to copy any RNA template in the presence of proper primers, but the authentic HCV 3' X tail sequence is the best substrate. The role of the C-terminal 21 amino acids of the protein in the course of replication is not clear at this time. This is the first report where a recombinant HCV replicase (either in full length or in C-terminally 21-amino-acid-deleted form) purified from bacteria is active on RNA substrates with an authentic 3' X-tail sequence in the absence of any primers. Many HCV proteins are active when purified from bacteria. The envelope proteins such as E1 and E2 needs proper glycosylation for its biological activity [9]. However, the glycosylation does not seem to play a crucial role in this case. Furthermore, this work shows that the C-terminal deleted form of the enzyme is still useful for the assay system which is important when considering that it is very hard to get the full-length protein due to the formation of inclusion bodies.

Recently, there has been a report on an HCV replicon that is capable of producing its RNA and nonstructural proteins in a cell culture system [11]. This is a major advance in HCV research. However, the system used in the current study is still a valuable tool for screening HCV replicase inhibitors since the initial screening can be accomplished in a test tube. Currently, no vaccine is available for HCV and the only therapy is using interferon-α, which has not been proved to be very effective. HCV replicase is a unique enzyme which is absent from normal host cell and is an essential factor for viral replication. Accordingly, it is a good target for the development of anti-HCV inhibitors. A large number of peptides, antibodies, and RNAs as well as synthetic chemicals are good candidates for HCV therapy. The *in vitro* assay methods described in this work can facilitate the initial screening of these candidate inhibitors.

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