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Virus-Free Synthesis of a Hepatitis C Virus P7 cDNA through a Three-Steps Polymerase Chain Reaction

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Abstract

Hepatitis C virus (HCV) infection represents a great public healthcare challenge as it affects nearly 170 million individuals worldwide. Therefore, the deep investigation of the mechanisms involved in the pathogenesis of chronic hepatitis induced by HCV is a crucial step in the design of novel targeted therapies for the treatment of this condition. However, techniques of molecular biology to characterize HCV proteins can suffer of intrinsic limitations due to high mutation rates of the virus genome.

In this study, we propose a novel strategy to synthesize a viral cDNA sequence corresponding to the p7 gene in HCV genome-free conditions. Our approach consists of a three-steps polymerase chain reactions (PCRs) by using a set of four large overlapping synthetic oligonucleotides aimed to separately amplify both 5' and 3' ends of the p7 gene; 5' and 3' products, overlapping themselves, were then used as a template in a third PCR amplification in order to get a full-length p7 cDNA.

Our methodology represents an interesting proof-of-principle as it allows for the safe manipulation of short viral genes. Moreover, this new technique overcomes the elevated genetic variability of HCV genomes without affecting the antigenic characteristics of the putative viral protein.

Keywords: HCV; p7; Polymerase chain reaction

Introduction and Study Design

Hepatitis C virus (HCV), a human pathogen affecting nearly 3% of the world's population [1], is the cause of chronic liver diseases that may lead to cirrhosis and hepatocellular carcinoma [2]. Techniques of molecular biology, such as polymerase chain reaction (PCR) and nucleotide sequencing, have been designed and widely used to characterize HCV RNA genome. Although these tests are highly sensitive, the results may be limited [2] due to HCV high mutation rates, low amount of RNA in improperly collected, handled and stored samples with a decreased accuracy of the methods [3]. Therefore, HCV RNA nucleic acid preparation (i.e. amplification) is a critical step in the molecular procedure [4].

HCV ability to shape its molecular architecture as well as to obtain a high genetic heterogeneity represents a challenge for the accurate establishment of molecular biological techniques [5]. Also, to highlight the viral elements responsible for virulence, accurate and greatly sensitive molecular techniques have to be carried out [5]. p7, a small integral protein orchestrating HCV virion assembly, plays a critical role in package of infectious viral progeny and it has been involved in genome replication [6]. Several Nuclear Magnetic Resonance Spectroscopy (NMR) studies, showed different potential topologies and conformations of p7 [7-10]. In particular, it has been demonstrated that p7 protein is organized as an N-terminal alpha-helix with two transmembrane segments (TMS1-2) connected by a short hydrophilic cytosolic segment [8]. Moreover, p7 forms cation-selective pores in the endoplasmic reticulum (ER) of the infected cell. p7 is also classified as a viroporin, due its ability to display ionic channel function; viroporins are a protein family able to manipulate membrane permeability to ions thus facilitating virus production [11].

However, while there is accumulating structural information on p7, there is not about how structural elements relate to its functions. p7 is characterized by an elevated genetic variability, a high content of hydrophobic amino acids as well as the small size; therefore, gene amplification and protein expression is difficult to achieve by well-known methodologies [12]. Different studies to obtain adequate p7 amount to determine functional and structural features have been carried out [13-20]. Nevertheless these studies revealed crucial steps, such as RNA instability and the presence of inhibitory substances, especially in biological samples, which represent the greatest problems to be overcome [4].

Here we propose a novel strategy based on a three-step PCR (PCR 1, PCR 2, and PCR 3) to generate HCV p7 amplification product (Figure 1A) in virus-free conditions aimed to reduce the impact of nucleic acid instability. Based on the p7 gene sequence (NCBI accession number NC_004102.1) we collectively designed two sets of oligonucleotides to generate a synthetic double-stranded p7 template of 195 bp including two artificial codons (an ATG and a stop codon at 5' and 3' of p7 sequence, respectively).

PCR 1 (Figure 1A) was aimed to generate an amplification product of 112 bp corresponding to position 1-112 of the p7 sequence. Primers oligonucleotides were designed as follows:

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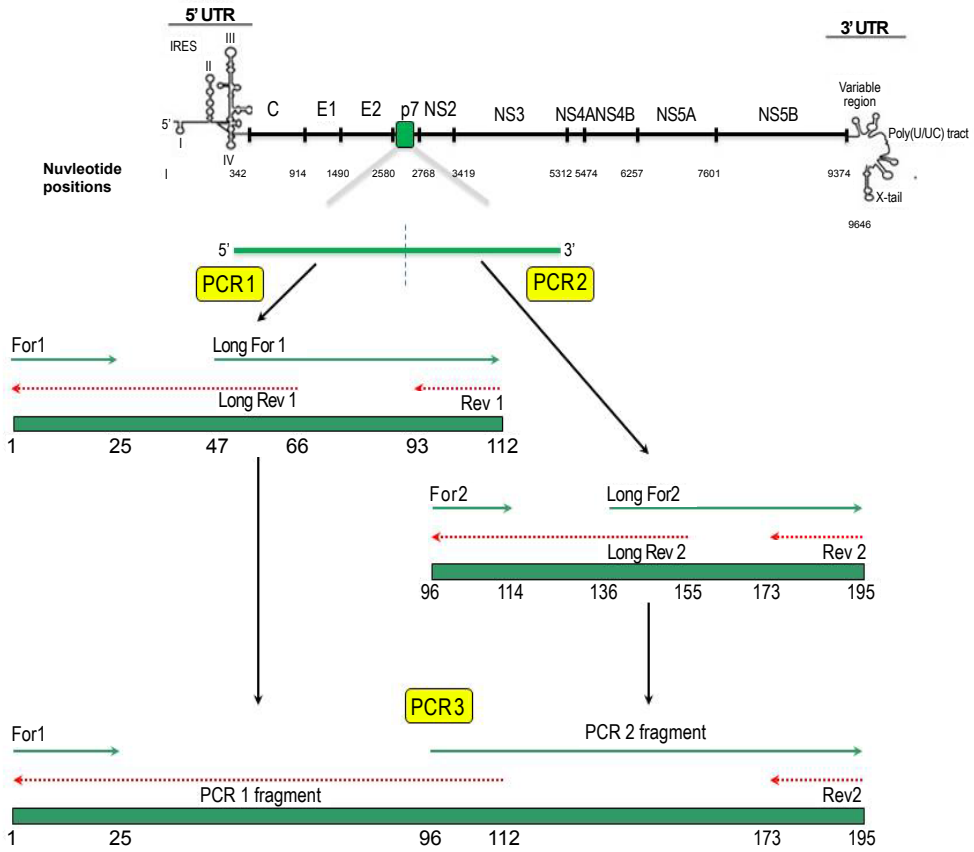

Figure 1A: Schematic representation of the methodology used to generate a p7 cDNA in a virus-free system (see text for details).

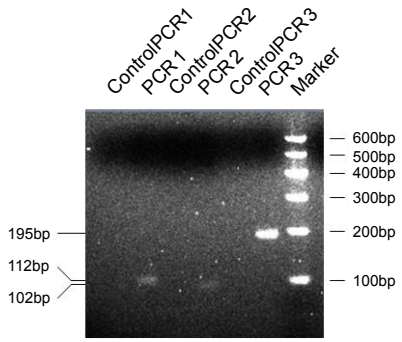

Figure 1B: Amplification products of 112 bp, 102 bp and 195 bp from PCR 1, PCR 2 and PCR 3, respectively. The fragment of 195 bp corresponds to the p7 full-length cDNA.

For 1 (pos. 1-25): 5'-ATg gCT TTg gAg AAC CTC gTA ATA C-3'

Long for 1 (pos. 47-112): 5'-ggA CgC ACg gTC TTg TgT CCT TCC TCg TgT TCT TCT gCT TTg CgT ggT ATC TgA Agg gTA ggT ggg-3'

Long Rev 1 (pos. 1-66): 5'-ggA CAC AAg ACC gTg CgT CCC ggC CAg ggA TgC TgC ATT gAg TAT TAC gAg gTT CTC CAA AgC CAT-3'

Rev 1 (pos. 93-113): 5'-CCC ACC TAC CCT TCA gAT AC-3'

Primers Long for 1 and Long Rev 1 were designed to have a significant overlapping sequence (19 nucleotides, ranging from position 47 to 66).

PCR 2 (Figure 1A) was aimed to generate an amplification product of 102 bp corresponding to position 93-195 of the p7 sequence. Primers oligonucleotides were designed as follow:

For 2 (pos. 96-114): 5'-TCT gAA ggg TAg gTg ggT g-3'

Long for 2 (pos. 136-195): 5'-TAC ggg ATg Tgg CCT CTC CTC CTg CTC CTg CTg gCg TTg CCT CAg Cgg gCA TAC gCA TAA-3'

Long Rev 2 (pos. 96-155): 5'-Agg AgA ggC CAC ATC CCg TAg AAg gCg TAg ACC gCT CCg ggC ACC CAC CTA CCC TTC AgA-3'

Rev 2 (pos. 173-195): 5'-TTA TgC gTA TgC CCg CTg Agg CA-3'

Primers Long for 2 and Long Rev 2 were designed to have a significant overlapping sequence (19 nucleotides as well, ranging from position 136 to 155).

Briefly, PCR 1 and 2 were performed in a thermocycler VERTI 96 well (Applied Biosystems) in a total volume of 50 µl containing 1 ng of each set of long oligonucleotides, 10 µM For 1 and Rev 1 primers, 1.25 U Taq DNA Polymerase (5 PRIME MasterMix, 2.5x), 50 mM KCl, 30 mM Tris-HCl, 1.5 mM Mg^{2+}, 0.1% Igepal-CA360 and 200 µM of each dNTP (*5 PRIME MasterMix, 2.5x*). Each PCR was carried out under the specific thermal cycler conditions. After reaction, the final products were separated on a 3% Agarose gel and visualized under UV light with ethidium bromide staining. We observed the amplification of two fragments of expected size (112 bp and 101 bp from PCR 1 and PCR 2, respectively) (Figure 1B).

PCR 3 (Figure 1A) was finally aimed to generate an amplification product corresponding to the p7 gene full-length (position 1-195). To

this purpose, we combined the amplification products from PCR 1 and PCR 2; these two fragments were designed in order to have a 16 bp overlapping sequence (position 96-112).

PCR 3 was carried out by using 1 ng of PCR 1 and PCR 2 products as template in 50 µL of the reaction mixture containing 10 µM For 1 and Rev 2 primers, 1.25 U Taq DNA Polymerase 50 mM KCl, 30 mM Tris-HCl, 1.5 mM Mg^{2+}, 0.1% Igepal-CA360 and 200 µM of each dNTP *(5 PRIME MasterMix, 2.5x)*. Thermal cycling was performed in a thermocycler VERTI 96well (Applied Biosystems) as described before.

The final product was separated on a 2% Agarose gel and visualized under UV light with ethidium bromide staining. As shown in Figure 1B, we observed a fragment of the expected size of 195 bp corresponding to the p7 full-length cDNA. Direct sequencing confirmed the accuracy of the procedure as well as the absence of random mutations (data not shown).

Conclusion

In conclusion, we propose an original proof-of-principle methodology to synthesize, by a three steps- polymerase chain reaction, a short viral cDNA in a virus-free system based on the design and combination of nucleotides covering the sequence of interest. This approach allows the advantage to work in a safe environment and to overcome the elevated genetic variability intrinsic to viral genomes without affecting the antigenic characteristics of the putative viral protein. More specifically, here we describe the development of this new approach by successfully synthesizing a cDNA corresponding to the HCV p7 sequence. Experiments are in progress in our laboratory to generate expression vectors carrying both wild-type and tagged p7 sequences to be used for the isolation of p7-interacting proteins in a proteomic-based approach [20] from liver eukaryotic cell systems. This will help us to shed light on the molecular mechanisms governing p7 activity in the pathogenesis of HCV-driven hepatitis.

Conflicts of interests

The authors declare that they have no competing financial interests.

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