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Development of the system ensuring a high-level expression of hepatitis C virus nonstructural NS5B and NS5A proteins

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Abstract

The plasmid pET-21d-2c-5BΔ55 effectively expressing a C-terminally truncated form (NS5BΔ55) of the hepatitis C virus (HCV) RNA-dependent RNA polymerase (RdRp) was constructed. It was derived from pET-21d-5BΔ55 plasmid and contained six mutations in the ATG-start codon region and an additional cistron upstream the target gene. The C-terminally His-tagged NS5BΔ55 protein was expressed in Rosetta(DE3) *Escherichia coli* strain bearing an additional pRARE plasmid encoding extra copies of rare tRNAs. The yield of the target enzyme exceeded by a factor of 29 the yield of NS5BΔ55 protein expressed from the parental pET-21d-5BΔ55 plasmid (5 mg/L). The increase in the protein yield could be explained by facilitated protein translation initiation, resulted from disruption of the stable secondary mRNA structure. The pET-21d-2c-5BΔ55 plasmid yielded one third amount of the protein when expressed in BL-21(DE3) strain, indicating that the pRARE plasmid is required for a high-level expression of NS5BΔ55 protein. The 29-fold enhancement of the protein yield was accompanied by only a 2.5-fold increase of the corresponding mRNA level. The expression of another HCV NS5A protein His-tagged at the C-terminus in the developed system yielded a similar amount of the protein (4 mg/L), whereas its N-terminally His-tagged counterpart was obtained in a 30 mg/L yield. The NS5A protein purified under denaturing conditions and renatured in solution inhibited the HCV RdRp and was a substrate for human casein kinase II.

Keywords: Hepatitis C virus; RNA-dependent RNA polymerase; High-level expression

The hepatitis C virus is the major etiological agent for sporadic and post-transfusion non-A non-B hepatitis [1]. It has infected about 170 million people worldwide. More than 80% of them develop chronic hepatitis that often causes liver cirrhosis and hepatocellular carcinoma. The studies of hepatitis C virus (HCV)[1] functioning as well as searching for potential antiviral drugs require substantial amounts of recombinant HCV proteins (see [2,3]).

The HCV genome is presented by a (+)-RNA of about 9600 nucleotides that encodes a single polyprotein [4], whose proteolysis results in 10 structural and nonstructural proteins. The NS5B protein is an RNA-dependent RNA polymerase (RdRp) [5,6], a key component of the HCV replication complex and a valuable target in the design of new antiviral agents [3].

RdRp was expressed in insect cells [3,7,8] or *Escherichia coli* [9–19]. Except for two cases [12,16], RdRp was expressed in bacterial systems under the control of *T7* promoter. The full-length RdRp protein tends to form inclusion bodies when expressed in *E. coli* due to a highly hydrophobic domain at the C-terminus of the polypeptide chain [9,11,13,15]. C-terminally truncated mutant forms NS5BΔ21 and NS5BΔ55 lacking 21 and 55 amino acid

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[1] *Abbreviations used:* RdRp, RNA-dependent RNA polymerase; HCV, hepatitis C virus; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; CKII, casein kinase II.

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residues, respectively, were obtained as soluble proteins [9–16,18,19]. The enzymatic activity of the NS5BΔ55 was 20 times higher then that of NS5BΔ21 [19]. Truncated RdRp mutant forms were obtained in bacterial expression systems in remarkably different yields. The yield of GST-NS5BΔ21 was reported to be 1 mg/L cell culture [9], whereas for NS5BΔ21-His it ranged from 0.6 [18] to 5 [15] and 6 mg/L [13]. It should be noted that most of the papers did not report the protein yield. Moreover, no yields were given for other NS5B forms, particularly NS5BΔ55 and NS5B-BLΔ8. Using the pET expression system, we obtained His-tagged NS5BΔ21 and NS5BΔ55 proteins in a yield of <1 mg/L [18]. Herein we describe an improved system providing a high-level expression of recombinant NS5BΔ55 and its regulator NS5A proteins in *E. coli* under the control of *T*7 promoter.

Materials and methods

Materials

DNA oligonucleotides were from Litech (Moscow, Russia) (Table 1). Restriction endonucleases and IPTG were from Sibenzyme (Novosibirsk, Russia), the pTTQ18 vector, T4 DNA ligase, T4 polynucleotide kinase, Pfu DNA polymerase, and heparin–agarose were from Amersham Biosciences (Little Chalfont, UK). Bacto triptone, yeast extract and bacto agar were from Difco (Detroit, MI). [α-^{32}P]UTP and [γ-^{32}P]ATP were (5000 Ci/mmol) from Izotop (Moscow, Russia). Ni–NTA–agarose resin, pET-15b and pET-21d vectors, and *E. coli* strains BL-21 {F− ompT hsdSB (rb− mB−) gal dcm}, BL-21(DE3) {F− ompT hsdSB (rb− mB−) gal dcm (DE3)}, Rosetta {F− ompT hsdSB (rb− mB−) gal dcm lacY1 pRARE6 (CmR)}, and Rosetta(DE3) {F− ompT hsdSB (rb− mB−) gal dcm lacY1 (DE3) pRARE6 (CmR)} were purchased from Novagen (Madison, WI). XL-1 Blue {*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*q*ZΔM15*Tn*10*(Tetr)]} *E. coli* strain, pTZ18R vector and RNAsin were purchased from Promega (Madison, WI). Other reagents of the highest purification grade were available from Sigma (St. Louis, MO) or Merck AG (Darmstadt, Germany). T7 RNA polymerase was expressed and purified as described [20]. The cloning and expression in *E. coli* of human casein kinase (CK II) will be described elsewhere.

The construction of NS5BΔ55 expression plasmids

The NS5BΔ55-coding DNA fragment lacking 55 amino acid residues was obtained by its amplification using oligos 1 and 2 and the pET-21d-5BΔ21 plasmid [18]. The PCR product was cloned into *Kpn*I and *Xho*I sites of pET-21d-5BΔ21 plasmid to give pET-21d-5BΔ55 plasmid. Oligos 3, 4, 5, and 6 were used to amplify the sequences bearing substitutions of six nucleotides upstream and downstream the ATG-start codon. The two resulting products corresponding to NS5B gene or the ribosome-binding site were digested with *Xba*I, *Eco*RI, and *Xho*I and cloned into *Xba*I and *Xho*I sites of the pET-21d vector to obtain the pET-21d-5BΔ55-TATGA plasmid. Oligos 7 and 8 were phosphorylated by T4 polynucleotide kinase, annealed with each other by heating at 94 °C, gradually cooled to 20 °C during 3 h, and cloned into *Xba*I and *Eco*RI sites in the

Table 1
Oligonucleotides used in this study

Oligo No.	Oligo name	Sequence
1	5B-Kpn	5′-TTACTCCAGGTGAGATC-3′
2	5B-Δ55	5′-ACGCCTCGAGGATTGGAGTGAGTTTGAGC-3′
3	T7	5′-TAATACGACTCACTATAGGG-3′
4	TATGA-For	5′-ATGAATTCGTCCTACACATGGACAGGC-3′
5	TATGA-Rev	5′-GAGAATTCATAGTATATCTCCTTCTTAAAGTTA-3′
6	5BR-Pst	5′-CGCCTTCGCCTTCATCTCC-3′
7	5B-2c-For	5′-CTAGAGGGTATTAATAATGTATCGATTAAATAAGGAGGAATAACATATG–3′
8	5B-2c-Rev	5′-AATTCATATGTTATTCCTCCTTATTTAATCGATACATTATTAATACCCT-3′
9	5B-lac-For	5′-GATCTAGAACCCCAGGCTTTACACTTTA-3′
10	5B-lac-Rev	5′-GATCTAGAGAAATTGTTATCCGCTCACAA-3′
11	5A-T-For	5′-CCCTTCTTAAGCTGCCAACGTGGTTACAAGGGTGTCT-3′
12	5A-T-Rev	5′-CGGGATCCTCGAGTTAGCAGCAGACGACGTCCTC-3′
13	5A-H-I	5′-CGAATTCTCGAGTCAGGTTCGTGGCTACGTGATGTTTGGGACTGGATTTGCACG GTGTTGACTGAC- 3′
14	5A-H-II	5′ TTCAAGACCTGGCTCCGTTCCAAGCTCCTGCCGCGTTTACCGGGTGTTCCGTTCTTA AGGATCCCG-3′
15	5A-H-III	5′-GGAGCCAGGTCTTGAAGTCAGTCAACACCGTG-3′
16	5A-H-For	5′-CGAATTCTCGAGTCAGGTTC-3′
17	5A-H-Rev	5′-CGGGATCCTTAAGAACGGAA-3′
18	5A-2cH-For	5′-ATGAATTCCAGCCATCATCATCATCAT-3′
19	5A-2c-For	5′-ATGAATTCTCAGGTTCGTGGCTACGT-3′
20	5A-2c-Rev	5′-ATCTCGAGGCAGCAGACGACGTCCTC-3′
21	(−)IRES-For	5′-TAATACGACTCACTATAGGGTGCACGGTCTACGAGACCT-3′
22	(−)IRES-Rev	5′-GCCAGCCCCCTGATGGGGGCGA-3′