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Primer Extension Analysis of HBV DNA with Strand-Specific Primers

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[Abstract] We performed primer extension assay to determine which steps of HBV DNA synthesis (*i.e.*, minus- and plus-strand DNA synthesis and circularization of RC DNA) are affected by phosphoacceptor site mutations in C protein. In these experiments, we used several specific oligonucleotide primers. For quantitation, the level of extended DNA (ED) was normalized to the level of a single internal standard (IS) DNA.

Materials and Reagents

1. Huh7 hepatoma cells (Japanese Collection of Research Bioresources Cell Bank, catalog number: JCRB0403)
2. Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gibco®, catalog number: 12800-017)
3. Fetal bovine serum (FBS) (Life Technologies, Gibco®, catalog number: 16000-044)
4. Penicillin/streptomycin (Life Technologies, Gibco®, catalog number: 15140-122)
5. OptiMEM (Life Technologies, Gibco®, catalog number: 31985-062)
6. 500 µl Opti-MEM (Life Technologies, Gibco®, catalog number: 31985-062)
7. PEG (USB, catalog number: 19959)
8. NaCl (Sigma-Aldrich, catalog number: S3014)
9. EDTA (Sigma-Aldrich, catalog number: E5134)
10. Polyethylenimine (Polysciences, catalog number: 23966)
11. Vent Exo (-) polymerase (New England Biolabs, catalog number: M0257S)
12. Micrococcal nuclease 1 µl (45 unit/µl) (Worthington Biochemical, I.U.B.: 3.1.31.1, catalog number: LS004798)
13. γ -32P-ATP (PerkinElmer Inc., catalog number: NEG035C)
14. T4 polynucleotide kinase (New England Biolabs, catalog number: M0201s)
15. Internal standard (IS) DNA (from HBV WT DNA Sac II/Xho I digested fragment) 1ng/1µl
16. 2.5 mM dNTP mixture (Takara Bio Company, catalog number: BH7901)
17. RNase A (Fermentas, catalog number: EN05331)
18. Tris-HCl (pH 8.8) (Sigma-Aldrich, catalog number: T6066)
19. $(NH4)_2SO_4$ (Sigma-Aldrich, catalog number: T6066)
20. KCl (Sigma-Aldrich, catalog number: P9541)

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21. $MgSO_4$ (Sigma-Aldrich, catalog number: 230391)
22. Triton X-100 (Sigma-Aldrich, catalog number: T8787)
23. Polyacrylamide (SERVA Electrophoresis GmbH, catalog number: 10687)
24. UREA (Duksan Hi-Metal, catalog number: CAS 57-13-6)
25. APS (Sigma-Aldrich, catalog number: A3678)
26. TEMED (Sigma-Aldrich, catalog number: T9281)
27. Boric Acid (Sigma-Aldrich, catalog number: B0394)
28. EDTA (Sigma-Aldrich, catalog number: E5134)
29. 1x DNA-containing reaction buffer (see Recipes)
30. 5% polyacrylamide gel (see Recipes)
31. 5x TBE (see Recipes)

Equipment

1. 10 cm dishes (Corning Incorporated, catalog number: 430167)

Software

1. Fujifilm Image Gauge software (version 4.0)

Procedure

1. Huh7 hepatoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin under a humidified atmosphere at 37 °C in 5% CO_2.
2. Cells were passaged every three days. 2 x 10^6 of Huh7 cells were seeded in 10 cm dish, one day before the transfection.
3. Next day, cells were (co)-transfected using polyethylenimine (PEI).
   PEI transfection method:
   a. In a sterile tube, total 10 µg of plasmid DNA (5 µg of phosphoacceptor site mutant and 5 µg of P-deficient mutant) was mixed with 500 µl Opti-MEM.
   b. Add 30 µl of PEI solution (1 µg/1 µl) to DNA-Opti-MEM solution and then vortex immediately.
   c. Incubate 15 min at room temperature.
   d. Then add PEI/DNA-Opti-MEM mixture to cells.
4. Transfection experiments were repeated at least three times.
5. Cytoplasmic core particles were prepared as previously described (Kim *et al.*, 2004). Three days after transfection, cells were used for core particle preparation.
   Cytoplasmic core particle preparations:
   a. Discard medium and wash with 10 ml PBS.

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b. Add PBS 1 ml, scrape the cells, and transfer to 1.5 ml tube.
c. Spin down at 13,000 rpm for 10 sec and discard supernatant.
d. Add 1 ml lysis buffer, vortex, and then incubate on ice for 10 min.
e. Spin down at 13,500 rpm for 2 min at 4 °C.
f. Transfer supernatant to fresh 1.5 ml tube.
g. Add micrococcal nuclease 1 µl (45 unit/µl), 1 M $MgCl_2$ 10 µl (final 10 mM), 1 M $CaCl_2$ 8 µl (final 8 mM).
h. Incubate 37 °C, 1 h.
i. Add 40% PEG 250 µl (26%), 5 M NaCl 100 µl (1.4 M), 0.5 M EDTA 118 µl (40 mM).
j. Put them in ice for 1 h.
k. Spin down at 13,500 rpm at 4 °C for 15 min.
l. Dissolve pellet in 20 µl nuclease free distilled water.

6. To analyze HBV DNA synthesis by primer extension analysis, HBV DNA was extracted from isolated core particles (Kim *et al.*, 2004).
   Prior to primer extension analysis, HBV DNA synthesis was analyzed by Southern blotting to see the levels of relaxed circular, double-stranded linear, and single-stranded DNAs (Jung *et al.,* 2014) (Figure 1).

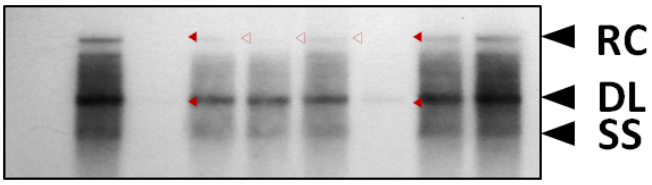

Figure 1. HBV DNA synthesis in core particles formed by STSSSS (WT) and mutant C proteins. To examine HBV DNA synthesis in core particles formed by phosphoacceptor site mutant C proteins, phosphoacceptor site mutant and P-deficient mutant were co-transfected into Huh7 cells. HBV DNA was extracted from isolated core particles (Kim *et al.*, 2004), and Southern blot analysis was performed (Jung *et al.,* 2014). Replicative-intermediate DNAs, relaxed circular (RC), double-stranded linear (DL), and single-stranded (SS) DNAs, are indicated.

7. Oligonucleotide DNA primers were 5'-end–labeled with 30 µCi γ-^{32}P-ATP at 37 °C for 3 h using T4 polynucleotide kinase.
   Labeling method

Primer 10 pmole/ul	2 µl
γ-^{32}P-ATP	3 µl
D.W	3 µl
10x T4 TNK buffer	1 µl
T4 polynucleotide kinase	1 µl

   The 5'-end–labeled primers HBV1665+ (5'-CTCTTGGACTCTCAGCAATGTCAAC-3'), HBV1744- (5'-CAGCTCCTCCCAGTCCTTAAACA-3'), and HBV1952- (5'-

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GAGAGTAACTCCACAGTAGCTCC -3') were used to measure the levels of the elongated minus-strand, plus-strand, and circularized RC DNAs, respectively.

HBV DNA (total 20 µl) extracted from core particles isolated from co-transfected Huh7 cells in 10-cm dishes was divided into four batches (5 µl): One batch for Southern blotting and three batches for primer extensions to measure minus-strand, plus-strand, and circularized RC DNAs.

For primer extension analyses of each C protein variant, 5ul viral DNA were heated to 95 °C for 5 min, treated with 1 U RNase A at 37 °C for 1 h, ethanol precipitated [ethanol precipitation: To 5 µl extracted DNA, add 195 µl distilled water and 20 µl 3 M sodium acetate, and mix by vortexing briefly. Add 440 µl 100% ethanol (molecular grade), vortex, and keep them overnight at -20 °C. Precipitate DNA by conventional method] and resuspended in distilled water (5 µl). End-labeled primers were extended with Vent Exo (-) polymerase, yielding products that annealed to the respective complementary HBV DNA sequences (Figure 2).

Viral DNA	5 µl
IS DNA (1 ng/µl)	1 µl
D.W	7 µl
DNA-containing reaction buffer	2 µl
2.5 mM dNTP	2 µl
Vent (-)	1 µl
Labeled primer	2 µl
Total	20 µl

Vortex and spin down
95 °C 1 min
95 °C 30 sec
60 °C 30 sec } 20 cycles
72 °C 30 sec
72 °C 1 min

8. 5 µl from 20 µl of the extended products were electrophoresed through 5% polyacrylamide gels containing 8 M urea (1,000 voltage 6 h). Gel was dried in gel dryer about 30 min at 60 °C. Dried gels were subjected to autoradiography (Figure 3), and relative levels of radioactivity were measured using the Fujifilm Image Gauge software, version 4.0.

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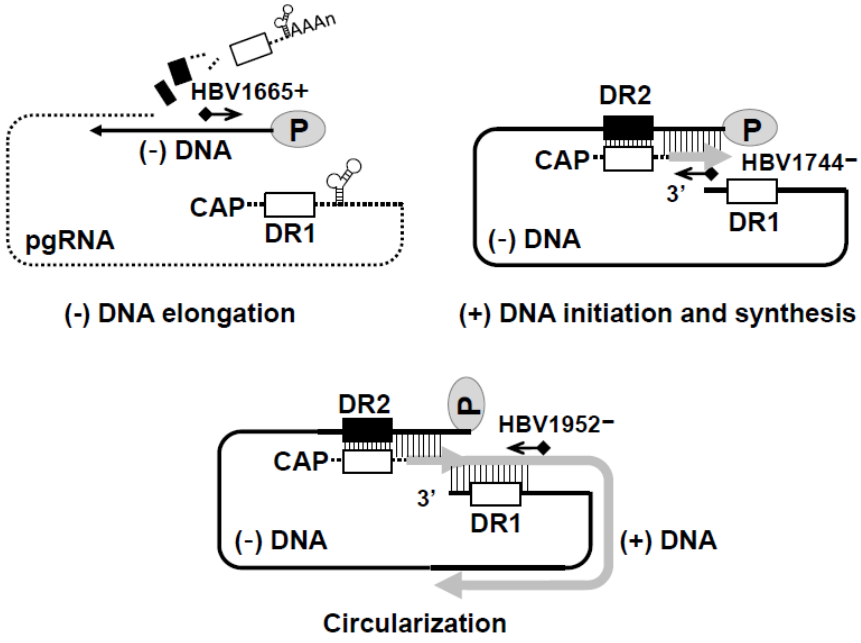

Figure 2. Schematic representation of oligonucleotides used for primer extension analysis. Minus- and plus-strand DNA elongation and circularization were detected using ^{32}P-end-labeled HBV1665+, HBV1744-, and HBV1952-, respectively.

Representative data

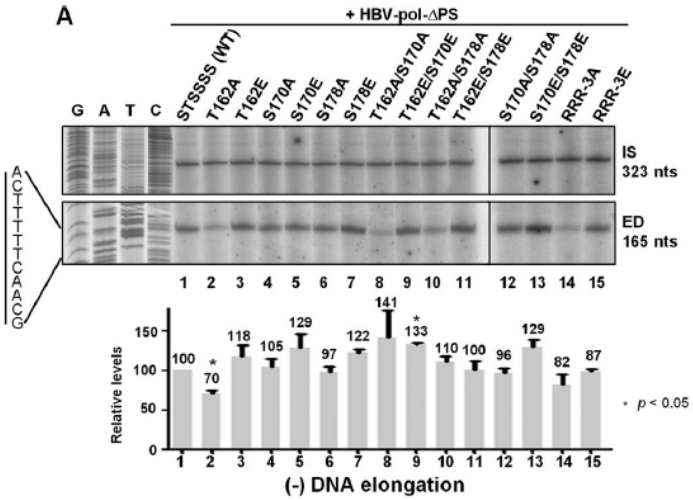

Figure 3. Minus-DNA elongation was detected using ^{32}P-end-labeled HBV1665+, showing that T162A exhibited the reduced minus-strand DNA elongation

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Recipes

1. Lysis buffer
   10 mM Tris (pH 8.0)
   1 mM EDTA
   0.2% NP40
   50 mM NaCl
2. 1x DNA-containing reaction buffer
   20 mM Tris-HCl (pH 8.8)
   10 mM $(NH_4)_2SO_4$
   10 mM KCl
   2 mM $MgSO_4$
   0.1% Triton X-100
3. 5% polyacrylamide gel

30% polyacrylamide	5.2 ml
5x TBE	6 ml
UREA	14.4 g
Add distilled water up to total	30 ml
10% AP	120 µl
TEMED	30 µl

4. 5x TBE

Tris-Hcl	54 g
Boric acid	28.5 g
0.5 M EDTA	20 ml
Add distilled water up	1 L
Total	1,000ml

Acknowledgments

This work was supported by National Research Foundation Grants funded by the Korean Government (NRF-2012-R1A2A2A01015370). We performed primer extension analysis by a previously described method (Lewellyn and Loeb, 2011), with minor modifications.

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