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Generation of Replication-Competent Hepatitis B Virus Genome from Blood Samples for Functional Characterization

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Abstract

Hepatitis B virus (HBV) infection can be associated with a spectrum of clinical outcomes. Transient transfection of the clinical HBV isolates in human hepatoma cell lines can establish their biological properties to shed light on their different pathogenic potentials, yet very few clinical HBV isolates have been functionally characterized so far. The technical challenges include faithful amplification of the full-length HBV genome from clinical samples and conversion into a replication-competent form. We have improved a published method to amplify the full-length HBV genome from blood samples. Two alternative approaches are used to render the cloned HBV genome replication competent: release and circularization of the 3.2-kb HBV genome prior to each transfection experiment or conversion of the monomeric clone into a tandem dimer version.

Keywords

HBV clinical isolates; Tandem dimer; Transfection

1 Introduction

1.1 Established Method to Amplify Full-Length HBV Genome from Blood Samples

So far at least eight hepatitis B virus (HBV) genotypes with nucleotide sequence divergence of 8 % or greater have been identified. They display distinct geographic distributions and clinical manifestations [1]. For example, infection with genotype C is associated with delayed seroconversion from hepatitis B e antigen (HBeAg) to anti-HBe [2] and increased risk to develop liver cirrhosis and hepatocellular carcinoma than genotype B infection [3]. Genotype C patients also respond less favorably to interferon therapy. In addition, the immune clearance phase of chronic infection selects for mutations in the precore, core promoter, and preS/S regions [4]. Comparison of the biological properties of different HBV mutants and genotypes requires the cloning of the entire viral genome, ideally from a large

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number of clinical samples to avoid bias [5, 6]. In this regard, the virion-associated HBV genome is relaxed circular and partially double stranded. Only the minus strand DNA is full length with the precore region present at both the 5' and 3' ends (Fig. 1). Twenty years ago, Gunther and colleagues developed a method to amplify the full-length HBV genome from blood samples by polymerase chain reaction (PCR) [7]. Both the sense and antisense primers target the precore region, which happens to be highly conserved among diverse HBV isolates.

1.2 HBV Genome Replication Requires Production of 3.5-kb Terminally Redundant Pregenomic RNA, Which Can Be Achieved by Circularization of the Full-Length HBV Genome

Upon infection of hepatocytes, virion-associated relaxed circular DNA is converted to covalently closed circular (ccc) DNA. The cccDNA in the nucleus serves as the template for transcription of coterminal 3.5-, 2.4-, 2.1-, and 0.7-kb RNAs. The 3.5-kb RNA is terminally redundant and has a heterogeneous 5' end, with the shorter version (pregenomic RNA or pg RNA) responsible for genome replication. First, it serves as the mRNA for translating both polymerase (P) and core proteins. Second, it is packaged into capsid particle assembled from core protein, where it is converted by co-packaged P protein into partially double-stranded DNA. In this regard, the full-length HBV genome amplified via the precore region is a linear molecule unable to produce the terminally redundant pg RNA. Gunther and colleagues solved this problem by adding at the 5' ends of both primers a recognition site for SspI, a class II-S restriction enzyme that cleaves downstream of the recognition site (Fig. 1) [7]. SspI-digested PCR product can be joined correctly by T4 DNA ligase to preserve the functionality of the circular HBV genome. They found replication of the SspI-digested PCR product in Huh7 human hepatoma cells even in the absence of in vitro ligation [7], possibly due to activity of a cellular ligase.

1.3 Use of Cloned Full-Length HBV Genome for Transfection: Problems and Solutions

The lack of a cloning step in the Gunther protocol makes the transfection work expensive and difficult to repeat. Besides, DNA circularization inside transfected cells is less efficient compared with in vitro ligation [8]. We added HindIII and SacI restriction sites (absent on the HBV genome) to the sense and antisense primers, respectively, to facilitate efficient cloning of the PCR product (Fig. 1) [5]. However, the use of cloned HBV genome for transient transfection poses novel problems. First, the mutation rate of the High Fidelity^{plus} DNA polymerase (Roche) used for PCR amplification [7] is only six times lower than that of Taq DNA polymerase. When it was employed to re-amplify the full-length HBV genome from a replication-competent HBV clone, five of nine PCR clones turned out to be defective in genome replication, protein expression, or both [8]. In contrast, the Phusion DNA polymerase (New England Biolabs) has 50 times lower mutation rate than Taq DNA polymerase, and all the ten PCR clones displayed comparable genome replication and protein expression [8]. Second, virion DNA from the same blood sample can display sequence heterogeneity, especially if collected at the immune clearance phase of chronic HBV infection. Two approaches can be used to overcome this problem. First, DNA from several PCR clones can be pooled for SspI digestion and selfligation. Second, after ligation of the HindIII/SacI double-digested PCR product with pUC18 DNA, the entire

transformation product can be grown directly in liquid culture to obtain plasmid DNA of the clone pool [8].

1.4 The 3.5-kb pg RNA Can Be Transcribed from Two Tandem Copies of the HBV Genome Cloned via the SphI Site in the Viral Genome

The need for SapI digestion of cloned HBV DNA followed by genome circularization introduces experimental variability. In this regard, the 3.5-kb terminally redundant pg RNA (around position 1818–1921) can be transcribed from two tandem copies of the HBV genome cloned to a vector via certain unique restriction sites on the viral genome, such as SphI (cleavage site at position 1238) (Fig. 1). Therefore, such tandem dimers can be used directly for transfection experiments without the need for enzymatic manipulation. A protocol to convert cloned monomeric HBV DNA into a replication-competent tandem dimer version is provided.

2 Materials

2.1 Viremic Serum Samples

Freshly obtained serum samples from HBV carriers are stored at 4 °C for several days prior to DNA extraction. Alternatively, they are stored at –80 °C for prolonged period of time.

2.2 Reagents

Chloroform.

Isoamyl alcohol.

Tris-saturated phenol.

QIAamp DNA Blood Mini Kit.

QIAquick PCR Purification Kit.

QIAquick Gel Extraction Kit.

QIAprep Miniprep Kit.

HiSpeed Plasmid Midi Kit.

dNTP.

Enzymes: BspQI, HindIII, SacI, ScaI, SphI, alkaline phosphatase, and Q5 DNA polymerase (New England Biolabs).

T4 DNA Ligase and DNA 3'-End Labeling Kit (Roche).

DH5α competent cells.

[α-³²P] dCTP.

PCR primers for amplification of the full-length HBV genome are sense 5' - CCGGAAAGCTTATGCTCTTCTTTTCACCTC TGCCTAATCA TC-3' (*Hind*III site underlined) and antisense 5' -CCGGAGAGCTCATGCTCTTCAAAAAGTTGCA TGGTGCTGGTG-3' (*Sac*I site underlined).

Oligonucleotide probe for screening SphI dimer has the sequence 5' - GCCATCAGCGCATGCGTGGAACCT-3' [9].

3 Methods

3.1 DNA Extraction

1. Mix 200 μ l serum sample with 20 μ l of protease and 200 μ l of Buffer AL, and incubate at 56 °C for 10 min.
2. Add 200 μ l of ethanol to precipitate DNA and pass the solution through QIAamp Mini spin column by centrifugation. Wash the column successively with buffer AW1 and AW2. After extra steps of spinning to remove residual liquid (*see Note 1*), elute retained DNA with 50 μ l of distilled water.

3.2 PCR Amplification for the Full-Length HBV Genome

1. In a specialized PCR hood or cell culture hood, set up a 50- μ l PCR reaction containing 0.5 mM each of the sense and antisense primers, 200 μ M of dNTP, 1x Q5 DNA polymerase buffer, 1 u of Q5 DNA polymerase (*see Note 2*), and 1–5 μ l of template DNA (*see Note 3*). Include a tube of PCR reaction lacking template DNA to serve as a negative control.
2. The PCR conditions are initial denaturation at 98 °C for 30 s, followed by 35–40 cycles of 98 °C for 10 s and 72 °C for 30 s, and final extension at 72 °C for 10 min. Run an aliquot of the PCR product (5 μ l) in 1 % agarose gel to verify successful amplification of the 3.2-kb HBV DNA (*see Note 4*).

3.3 Cloning of the PCR Product to Generate Monomeric HBV Construct

1. Run the remainder of the PCR product in 1 % agarose gel to separate the 3.2-kb HBV DNA from primer dimers. Cut out the 3.2-kb band, and extract DNA from gel slice using QIAquick DNA extraction kit. Elute DNA in 50 μ l of water.
2. Digest eluted HBV DNA and 2 μ g of pUC18 plasmid DNA at 37 °C for >2 h with 20 u each of *Hind*III and *Sac*I, and purify both HBV and pUC18 DNA through QIAquick PCR purification column according to manufacturer's manual. Elute DNA in 30–50 μ l TE (pH 8.0) buffer. Measure DNA concentration using Nanodrop 2000c spectrophotometer.
3. Ligate HBV DNA with pUC18 DNA in a 10- μ l volume using 3:1 molar ratio (about 65 ng of HBV DNA with 20 ng pUC18 DNA), at 14 °C overnight. Set up a tube of pUC18 DNA selfligation to serve as a negative control.
4. Incubate 5 μ l of the ligation product with 50 μ l of competent DH5 α cells on ice for 30 min, followed by heat shock at 37 °C for 45 s. Let stand on ice for 2 min

and add 500 μ l of LB medium. Shake at 37 °C for 30 min and spread 50 and 200 μ l each onto an LB plate containing 100 μ g/ml ampicillin. Incubate the plates at 37 °C overnight (*see Note 5*).

5. Pick up five or more well-separated single colonies from the plates and grow them overnight in 5 ml of LB medium supplemented with 100 μ g/ml of ampicillin. Extract plasmid DNA using QIAprep Miniprep Kit, followed by measurement of DNA concentration. Double digest 0.5–1 μ g of plasmid DNA with HindIII and SacI, followed by gel electrophoresis. Recombinant plasmids should contain a 3.2-kb band in addition to a band of 2.7 kb (pUC18).
6. If necessary, determine the nucleotide sequence of the cloned HBV DNA by customer sequencing. About 5–6 overlapping sequencing reactions are needed to cover the entire HBV genome.

3.4 Preparation of Circularized HBV Genome for Transfection Experiments

1. Digest recombinant plasmid at 37 °C for at least 4 h with BspQI (*see Note 6*) and ScaI (*see Note 7*). Run the DNA digest in 1 % agarose gel and cut out the 3.2-kb band. Extract DNA using QIAquick DNA extraction kit and elute DNA in 50 μ l TE buffer.
2. Measure DNA concentration using Nanodrop spectrophotometer. Ligate at 14 °C overnight the full-length HBV genome with T4 DNA ligase at a low concentration of 0.5 ng/ μ l to promote intramolecular ligation. Extract the ligation product sequentially with equal volumes of Tris-HCl (pH 8.0) saturated phenol and chloroform/isoamyl alcohol (24:1), and precipitate DNA with two volumes of ethanol in the presence of 300 mM sodium acetate, pH 5.2. Store the Eppendorf tubes at –20 °C overnight.
3. Centrifuge the Eppendorf tubes in the cold room at 14,000 x g for 30 min. Wash the DNA pellet with cold 70 % ethanol followed by another wash with cold pure ethanol. Let air dry, and dissolve the purified HBV DNA in TE buffer (pH 8.0) at 0.2–0.5 μ g/ μ l for transfection experiment.

3.5 Conversion of Monomeric HBV DNA Construct into SphI Dimer

1. Digest the circularized full-length HBV DNA described above with SphI. Also digest 2 μ g of pUC18 DNA with SphI to completion, followed by treatment with alkaline phosphatase to prevent self-ligation.
2. Ligate the SphI cut HBV DNA with SphI cut, dephosphorylated pUC18 DNA at 10:1 molar ratio [9] (*see Note 8*). Perform pUC18 self-ligation to serve as a negative control. Transform competent DH5 α cells with the ligation products and spread out E. coli on LB/ampicillin plates.
3. Pick up individual colonies from the pUC18 + HBV plates and regrow them on gridded nylon membrane placed on top of LB/ampicillin plates. After overnight growth, make a membrane lift (use a semi-wet membrane) to transfer a fraction of bacteria to another disc of nylon membrane (*see Note 9*). Place the disc on top

of three layers of Whatman paper wetted with denaturation solution (0.5 N NaOH/1.5 M NaCl) and let stand for 5 min. Next, transfer the disc on top of neutralization solution (0.5 M Tris-HCl, pH 7.5/1.5 M NaCl). Let float for 10 min and then shake several times to let it submerge into the solution. Rinse briefly in 2×SSC solution and let air dry.

4. Prehybridize the lift at 60 °C for 2 h in a solution containing 6×SSC/0.1 % SDS, and 100 µg/ml sheared and denatured salmon sperm DNA. Meanwhile, label 20 pmol of the oligonucleotide 5'-GCCATCAGCGCATGCGTGGAACCT-3' with 5 µl of [α -³²P] dCTP (10 mCi/ml) using a DNA 3'-End Labeling Kit (Boehringer Mannheim), at 37 °C for 1 h. Replace with fresh prehybridization solution, and add ³²P labeled oligonucleotide probe at 5–10 × 10⁵/ml. Hybridize at 60 °C for 2 h. Wash the membrane sequentially with 6×SSC/0.1 % SDS and 2×SSC/0.1 % SDS, at 60 °C for 10 min. Expose to an X-ray film.
5. Pick up several hybridization positive colonies from the master plate and grow them into 5-ml liquid culture. Perform plasmid preparation and digest the miniprep DNA with a panel of restriction enzymes including HindIII, SphI, and BglII (or RsrII, XbaI), followed by electrophoresis in 0.8 % agarose gel. HindIII has a single cleavage site on pUC18 but none on HBV and will generate a single band of 9.1 kb for a dimer. SphI should generate two bands: 2.7 and 3.2 kb, with the 3.2-kb band twice stronger than that of the 2.7-kb band for a dimer. Both BglII and RsrII have a single cleavage site on the HBV genome but none on the pUC18 vector and will generate a 3.2-kb band and a 5.9-kb band if the dimer is tandem (tail to head). A tandem SphI dimer will also generate a 3.2-kb band by XbaI digestion, although the remaining DNA is cleaved into two fragments due to the presence of an XbaI site on the polycloning site of pUC18.

4. Notes

1. Make sure that liquid is completely spun out of the column before elution. Otherwise residual ethanol will inhibit subsequent PCR reaction.
2. Q5 DNA polymerase has 100 times lower mutation rate than Taq DNA polymerase and thus has higher fidelity than Phusion DNA polymerase.
3. Observe all precautions to minimize contamination, including UV treatment of the hood, cotton tips, adding template DNA at the very last step, and strict separation of the pre-and post-PCR areas and equipments.
4. Successful amplification of the full-length HBV genome from blood samples is dependent on high viremia titer. For low viremic samples, the HBV genome could be amplified as two overlapping DNA fragments (as shorter DNA fragments are much easier to amplify than the full-length genome), followed by their joining by overlap extension PCR. A concern is that the full-length genomes thus generated may not derive from the same parental molecules.

5. Alternatively, the transformation product is transferred to 20 ml of LB medium supplemented with 100 µg/ml of ampicillin and shaken at 37 °C overnight. The plasmid DNA thus extracted will represent the clone pool.
6. BspQI is an isozyme of SapI, but about half the price.
7. Scal converts the 2.7-kb pUC18 DNA into smaller 1.8-and 0.5-kb fragments, thus facilitating unambiguous recovery of the 3.2-kb HBV DNA.
8. A high insert/vector ratio is needed to promote joining of more than one copies of the HBV genome to the vector. The yield of dimer could be increased by ligating HBV DNA alone for 5–10 min prior to addition of pUC18 DNA. In case this approach fails to generate tandem dimer, the SphI monomer clones thus obtained can be used to obtain large amount of HBV DNA linearized at the SphI site for dimer construction. This will facilitate dimer construction.
9. Alternatively, spike the bacteria into duplicate petri dishes and use the membrane from one dish for hybridization experiment.

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Flow chart for the generation of replication-competent HBV genomes from blood samples. Virion-associated HBV DNA has the minus strand DNA (*thick line*) complete. The sense (S) primer anneals to its 3' end to generate full-length plus strand, which will serve as the template for the antisense (AS) primer to generate more minus strand DNA. The HindIII and SacI sites introduced to the sense and antisense primers, respectively, will allow efficient cloning of the PCR product to pUC18 vector, whereas the internal BspQI sites allow subsequent precise release of the HBV genome. Such a linear HBV genome can be ligated in vitro to make it replication competent (capable of producing the terminally redundant pg RNA), or the ligated DNA is further digested with SphI and ligated with SphI cut, dephosphorylated pUC18 DNA. Bacterial colonies harboring tandem SphI dimer can be screened by hybridization with an oligoprobe spanning the SphI site. The 3.5-kb pg RNA can be produced from such a tandem dimer construct