Improved Method for Rapid and Efficient Determination of Genome Replication and Protein Expression of Clinical Hepatitis B Virus Isolates[▽]

Yanli Qin,^{1,2} Jiming Zhang,^{2*} Tamako Garcia,¹ Kiyoaki Ito,¹ Danielle Gutelius,¹
Jisu Li,¹ Jack Wands,¹ and Shuping Tong^{1*}

Liver Research Center, Rhode Island Hospital, Brown University, Providence, Rhode Island 02903,¹ and Department of Infectious Diseases, Huashan Hospital, Shanghai Medical College, Fudan University, Shanghai 200040, China²

Received 21 November 2010/Returned for modification 6 January 2011/Accepted 21 January 2011

Different hepatitis B virus (HBV) genotypes and variants are associated with different clinical outcomes and/or response to antiviral therapy, yet the comparison of the *in vitro* replication capacity of a large number of clinical isolates remains technically challenging and time-consuming. Although the full-length HBV genome can be amplified from high-titer blood samples by PCR using *High Fidelity^{plus}* DNA polymerase and primers targeting the conserved precore region, the HBV clones thus generated are replication deficient due to the inability to generate the terminally redundant pregenomic RNA essential for genome replication. The transfection experiment is further complicated by PCR errors and the presence of viral quasispecies. A previous study found that the precise removal of non-HBV sequence by *SapI* digestion led to HBV replication in transfected cells, possibly due to low-level genome circularization by a cellular enzyme. We released HBV genome from the cloning vector using *BspQI*, an inexpensive isoschizomer of *SapI*, and increased the efficiency of genome replication by an extra step of *in vitro* DNA ligation. The uncut plasmid DNA can be used for transfection if the sole purpose is to study envelope protein expression. We found significant PCR errors associated with the *High Fidelity^{plus}* DNA polymerase, which could be greatly diminished using *Phusion* DNA polymerase or masked by the use of a clone pool. The reduced PCR error and modified enzymatic steps prior to transfection should facilitate a more widespread functional characterization of clinical HBV isolates, while the clone pool approach is useful for samples with significant sequence heterogeneity.

Hepatitis B virus (HBV) can be classified into eight genotypes with a minimum sequence divergence of 8% at the nucleotide level. These genotypes circulate in different parts of the world and are associated with different courses of infection and response to therapy (5, 20, 21, 23). Furthermore, genetic variants can be selected at the late stage of chronic infection (such as precore and core promoter mutants), by vaccination (immune escape mutant), or following therapy with nucleoside analogues (drug-resistant mutants) (3, 4, 22, 34). From a clinical point of view, certain HBV isolates have been associated with fulminant hepatitis with a high mortality rate (19), whereas other strains are linked to occult HBV infection (25). Understanding the molecular basis for diverse outcomes of HBV infection requires the cloning of the 3.2-kb genome from clinical samples, followed by its functional characterization through transfection experiments. In this regard, blood is a more accessible source of the HBV genome than the liver. Virion-associated HBV DNA consists of a full-length minus strand and a plus strand of variable lengths, and it has a relaxed circular configuration (Fig. 1). Fifteen years ago, Günther and

colleagues developed a method to amplify the full-length HBV genome using a primer pair targeting the highly conserved precore region, which happens to be present at both the 5' and 3' ends of the minus-strand DNA (12). We have slightly modified the PCR primers such that the unique restriction sites incorporated into the sense and antisense primers permit the directional cloning of the PCR product (24).

A single copy of the HBV genome inserted into a cloning vector via the precore region is, however, unable to express certain viral proteins due to the disruption of the coding sequence by the HBV-vector junctions. It cannot replicate because of the inability to produce the terminally redundant (3.5-kb) pregenomic RNA (pg RNA). During the natural course of HBV infection, viral protein expression and genome replication originate from the covalently closed circular DNA (ccc DNA) in the nucleus, which is derived from virion-associated DNA by a series of enzymatic reactions. Several co-terminal mRNAs are transcribed from the ccc DNA under various promoters. The pre-S promoter directs the synthesis of the 2.4-kb mRNA for the translation of the large (L) envelope protein, while the S promoter is responsible for the production of the 2.1-kb mRNA for the middle (M) and small (S) envelope proteins. The core promoter drives the transcription of two terminally redundant 3.5-kb mRNAs: the precore RNA for HBeAg and the shorter pg RNA for core protein and DNA polymerase. In addition, the pg RNA is packaged with DNA polymerase into a nascent core protein particle, where it is converted to double-stranded DNA through a series of enzy-

* Corresponding author. Mailing address for S. Tong: Liver Research Center, 55 Claverick Street, 4th Floor, Providence, RI 02903. Phone: (401) 444-7365. Fax: (401) 444-2939. E-mail: Shuping_Tong_MD@Brown.edu. Mailing address for J. Zhang: Department of Infectious Diseases, Huashan Hospital, 12 Middle Wulumuqi Road, Shanghai 200040, China. Phone: 86-021-52888125. Fax: 86-021-62486140. E-mail: Jmzhang@fudan.edu.cn.

[▽] Published ahead of print on 2 February 2011.

FIG. 1. Generation of replication-competent HBV forms from full-length PCR product. Primers targeting the precore region permit the amplification of the full-length HBV genome from virion-associated DNA. The incorporation of HindIII and SacI sites into the two primers enables directional cloning. The replication of the HBV genome requires the transcription of the 3.5-kb terminally redundant pg RNA under the core promoter (shown in a black oval), which is feasible from the ccc DNA template but not from a single copy of the HBV genome cloned to a vector. One approach is to release the HBV insert by digestion with BspQI, followed by the construction of an EcoRI dimer through an intermediate of the precore dimer. A much simpler approach is to use the BspQI digest, which is converted to a circular form by a cellular ligase or, more efficiently, by T4 DNA ligase *in vitro*.

matic reactions. Therefore, pg RNA is essential for HBV genome replication. While the circular nature of ccc DNA permits the transcription of the genome-length precore RNA and pg RNA (Fig. 1), the generation of such RNAs from vector-associated HBV DNA requires the duplication of at least a fraction of the viral genome. A common strategy is to convert a monomeric HBV DNA construct into a tandem (tail-to-head) dimer using a unique restriction site on the viral genome (such as EcoRI for genotype A or SphI for many genotypes), but the procedure is quite labor-intensive (30).

The construction of a tandem dimer via the precore region is hampered by the lack of a unique restriction site at this location. Günther and colleagues solved this problem by introducing a recognition sequence for SapI, a class II-S restriction enzyme, into both PCR primers (12). Unfortunately, the precore-based dimer with this particular insert-vector junction still cannot produce the pg RNA (although a trimer can) (Fig. 1), necessitating its further conversion into an EcoRI or SphI dimer. Considering the complexity in making stable HBV constructs, Günther and colleagues directly transfected SapI-digested PCR product, which contains the entire HBV genome without any foreign sequence, into the Huh7 human hepatoma cell line. HBV DNA replication, at a much reduced level compared to that of the corresponding dimer cloned via the EcoRI site (the EcoRI dimer), was observed (12). In the present study, we modified the Günther method for the rapid, efficient, and cost-effective characterization of the biological properties of blood-derived HBV isolates.

MATERIALS AND METHODS

HBV DNA constructs. The 2A and 4B genomes are available as tandem EcoRI dimers in the pUC18 vector (24). The C⁻ and P⁻ constructs of clone 4B contain nonsense mutations in the core and polymerase genes, respectively (32). EcoRI monomers in the pUC18 vector were generated in the present study, and plasmid DNA was prepared using a Hispeed plasmid Midikit (Qiagen). The HBV sequence was reamplified from 10 ng of 2A and 4B clones by 30 cycles of PCR using sense primer PC1 (5'-CCGAAAGCTTATGCTCTTCTTTTACCTC TGCCTAATCATC-3'), antisense primer PC2 (5'-CCGAGAGCTCATGCTC TTCAAAAGTTGCATGGTGCTGGTG-3'), and *High Fidelity^{plus}* DNA polymerase (Roche) as detailed elsewhere (24). Alternatively, clone 4B was reamplified by 1 U of *Phusion* DNA polymerase (New England BioLabs) in a 50- μ l volume containing 10 ng of template DNA, 200 μ M deoxynucleoside triphosphate (dNTP), and 1 mM (each) PC1 and PC2 primers. The cycling conditions for *Phusion* polymerase consist of initial denaturation at 98°C for 30 s, followed by 30 cycles of 98°C for 10 s, 70°C for 10 s, and 72°C for 30 s, and then a final extension at 72°C for 10 min. The PCR product generated by either polymerase was double digested with HindIII and SacI and ligated to the same sites of a modified pUC18 vector with the original SapI restriction site (position 683) destroyed. To obtain individual clones, the transformation product was spread to ampicillin-containing LB plates as usual. For the population approach, the transformation product was shaken at 37°C for 30 min in 0.5 ml of LB medium, followed by overnight growth in 100 ml of medium supplemented with ampicillin. The problem of colonies arising from vector self-ligation was minimized by two rounds of digestion of vector DNA with HindIII and SacI, as well as further digestion with a third enzyme (SphI) located between the HindIII and SacI sites on the polylinker.

DNA preparation prior to transfection. A single copy of the full-length HBV genome was released by the EcoRI digestion of the EcoRI monomer, ApaI or SphI digestion of the EcoRI dimer, and digestion of the monomeric PCR clones or clone pools at 50°C with BspQI (New England BioLabs). The digested DNA was purified through QIAquick PCR purification columns (Qiagen) and resuspended in endotoxin-free Tris-EDTA buffer. Alternatively, the 3.2-kb HBV DNA was eluted from the agarose gel. To circularize the HBV genome with minimum intermolecular ligation, 1.5 μ g of the digest or gel-purified HBV DNA was ligated at 16°C overnight with 1,600 U (4 μ l) of T4 DNA ligase (New England BioLabs) in a total volume of 1.5 ml. The ligation product was extracted sequentially with phenol and chloroform-isoamyl alcohol (24:1), precipitated with isopropanol in the presence of glycogen, washed with 70% ethanol, and resuspended in 10 to 15 μ l of endotoxin-free TE buffer. To remove the sticky EcoRI ends of linear HBV genome, the EcoRI digest was treated with Klenow fragment at 1 U/ μ g DNA. Before transfection, DNA concentration was determined by spectrometry and confirmed by running an aliquot in agarose gels.

Transient transfection and analysis of genome replication and virion secretion. Huh7 cells were seeded in six-well plates and transfected by TransIT-LT1 reagent (Mirus) using the same amount of 3.2 kb HBV DNA. The total amount of DNA transfected was kept constant by adding various amounts of the pUC18 DNA. Cells and culture supernatant were harvested at day 5 posttransfection. Replicative HBV DNA was extracted from intracellular core particles (9, 32). Virus particles were immunoprecipitated from culture supernatant with a horse polyclonal anti-HBs antibody (Ad/Ay; Novus). Both replicative DNA and virion DNA were subject to Southern blot analysis with the full-length 4B DNA as a probe.

Analysis of protein expression and secretion. Intracellular envelope proteins were detected by Western blot analysis as described previously (9, 10, 15). The dilutions for the primary and secondary antibodies were 1:5,000 for a horse polyclonal anti-HBs (Ad/Ay; Novus), 1:100,000 for rabbit anti-horse antibody conjugated with horse radish peroxidase (HRP) (Abcam), 1:1,000 for a mouse anti-pre-S2 antibody (Virogen), 1:40,000 for HRP-conjugated anti-mouse antibody, and 1:10,000 for mouse antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (32). HBeAg was immunoprecipitated from 0.6 ml of precleared culture supernatant with 1.5 μ l of a polyclonal rabbit anti-core antibody (Dako) and revealed by Western blotting using the same antibody (14). The core protein present in cell lysate was detected by the same procedure. In addition, secreted HBeAg and HBsAg were quantified using enzyme-linked immunosorbent assay (ELISA) kits from DiaSorin and Abzyme, respectively.

RESULTS

Rationale. We employed clones 2A and 4B of genotype A to systemically test the impact of the DNA format on efficiencies

FIG. 2. Replication capacity of the HBV genome linearized by *ApaI* and *SphI* restriction enzymes. The *EcoRI* dimer of clone 4B was digested with *ApaI* or *SphI*, with or without further treatment with T4 DNA ligase before transfection into Huh7 cells. The uncut dimer served as a positive control. Intracellular replicative DNA was analyzed by Southern blot analysis. The *EcoRI* and *EcoRI*-*RsrII* double-digested HBV DNA served as markers of 3.2 and 1.7 kb/1.5 kb (lane 1). PDS, partially double-stranded DNA form; SS, single-stranded DNA form.

of genome replication and protein expression and to evaluate the functional consequence of PCR errors. Both clones have been completely sequenced and extensively characterized (15, 17, 24, 32). Clone 4B has much higher replication capacity than clone 2A but much lower HBeAg expression due to the presence of a quadruple core promoter mutation (24, 32). Its core protein and HBeAg were detected less efficiently by a polyclonal anti-HBc antibody (Dako) used in Western blot analysis due to an E77Q mutation in the core gene (32). Moreover, it has an extra glycosylation site in the S domain, thus generating additional forms of L, M, and S envelope proteins (15, 17). We will focus on clone 4B when discussing the replication phenotype and on clone 2A for protein expression.

Replication capacity of linear forms of full-length HBV genome and improvement by *in vitro* circularization. Due to the terminal redundancy in pg RNA, HBV DNA replication can be initiated from a circular genome (ccc DNA) or an overlong genome, such as a vector-linked *EcoRI* dimer. The fact that Günther and colleagues could detect HBV genome replication from a linear template (12) is somewhat surprising. The most plausible explanation is that a fraction of transfected DNA was ligated by a cellular enzyme to form a ccc DNA equivalent. If this is the case, an HBV genome linearized at other positions also should support DNA replication. The digestion of an *EcoRI* dimer of clone 4B with single-cutting restriction enzymes *ApaI* (at position 2600) and *SphI* (at position 1232) releases one copy of the HBV genome. Both *ApaI* and *SphI* digests were replication competent in Huh7 cells, although at much reduced levels compared to that of the uncut dimer (Fig. 2, compare lanes 2 and 3 with lane 6). Interestingly, the treatment of the digests with T4 DNA ligase at a low DNA concentration (1 μ g/ml) to promote intramolecular ligation, followed by DNA concentration, significantly enhanced replication capacity (lanes 4 and 5). This finding suggests that the ligation of linear HBV DNA is far more efficient by T4

DNA ligase *in vitro* than by a host enzyme within cellular milieu.

Release of the HBV genome from an *EcoRI* monomer is required for genome replication and efficient protein expression. To determine whether the findings described above can be extended to monomeric constructs, we generated *EcoRI* monomers for both 2A and 4B genomes. The uncut *EcoRI* monomer of clone 4B was replication deficient (the faint bands in lane 8 of Fig. 3C ran at aberrant positions). The release of the 4B genome by *EcoRI* digestion led to genome replication (Fig. 3C, lane 9). The treatment of the *EcoRI* digest with T4 DNA ligase prior to transfection further increased genome replication, close to the level of the *EcoRI* dimer (lanes 10 and 12). In contrast, blunting the *EcoRI* ends with Klenow fragment, which is expected to reduce the efficiency of DNA ligation and will induce frameshift mutations into the polymerase gene when the blunted genome is circularized, nearly abolished genome replication (lane 11).

The *EcoRI* monomer maintains the undisrupted precore/core gene and its upstream core promoter (Fig. 3A). Indeed, the uncut *EcoRI* monomer can express low levels of core protein (data not shown) and HBeAg (Fig. 3F, lane 1). HBeAg production was enhanced by *EcoRI* digestion and further increased by an additional step of ligation by T4 DNA ligase (lanes 2 and 3). The expression of the three envelope proteins was absent or negligible with the uncut *EcoRI* monomer (Fig. 3E, lane 1), because the *EcoRI* junction interrupts the S promoter and coding sequences for L and M proteins (Fig. 3A). *EcoRI* digestion greatly improved S protein expression (lane 2), although efficient M and L protein expression required an additional step of DNA ligation (lane 3). The treatment of the *EcoRI* digest with Klenow fragment, which will introduce a frameshift mutation into the pre-S2 region of the enveloped gene in the circularized HBV genome, abolished M and L protein expression (lane 4). The results are summarized in Fig. 3B.

Release of the HBV genome from the full-length PCR clones is required for genome replication and HBeAg expression but not for envelope protein expression. To improve the Günther method for the functional characterization of clinical HBV isolates, we reamplified the HBV genome from clone 4B using primers targeting the precore region and *High Fidelity^{plus}* DNA polymerase. The resultant PCR clones in the pUC18 vector have truncated HBeAg coding sequence and the separation of the core promoter from the core gene (Fig. 4A). This can explain defective genome replication and HBeAg expression for progeny clones 1 and 9 when the uncut plasmid DNA was transfected (Fig. 4B and D, lanes 1 and 4). The release of the full-length HBV genome by *BspQI* digestion led to genome replication and HBeAg expression (lanes 2 and 5), which could be markedly enhanced by an extra step of *in vitro* DNA ligation (lanes 3 and 6). In contrast to the precore/core gene, the envelope gene is undisrupted in the PCR clones and preceded by the L and S promoters (Fig. 4A). In fact, the uncut plasmid DNA expressed much higher levels of L, M, and S proteins than the *BspQI* digest whether or not the HBV genome was further circularized by T4 DNA ligase (Fig. 4C, compare lanes 1, 2, and 3). It is remarkable that the levels of the three envelope proteins also were much higher than that achieved by the corresponding *EcoRI* dimer (lane 7). Therefore, the clon-

FIG. 3. Genome replication, virion secretion, and viral protein expression initiated from EcoRI monomers. (A) Schematic representation of the EcoRI monomer with vector sequence shown as slashed lines. Positions of L, S, and core (C) promoters and coding sequences are indicated. (B) Summary of protein expression and genome replication from the EcoRI monomer that was undigested (M), EcoRI digested (M+E), EcoRI digested and T4 DNA ligase treated (M+E+T), or EcoRI digested and Klenow fragment treated (M+E+K). (C) Intracellular replicative DNA. D, dimer; C⁻ D, core-minus dimer as a negative control for DNA replication; P⁻ M, polymerase-minus monomer as another negative control. (D) Extracellular virion DNA. (E) Intracellular L, M, and S envelope proteins and GAPDH as a loading control. Only the two size forms found in clone 2A are indicated: gp42 and p39 (L), gp36 and gp33 (M), and gp27 and p24 (S). (F) Extracellular HBeAg measured by ELISA (based on three transfections), using 1 μ l supernatant for 2A constructs and 2 μ l for 4B constructs. Values from 2A dimers were set at 100%.

ing of the full-length HBV genome via the precore region gives rise to extremely efficient envelope protein expression driven by endogenous enhancer/promoter elements. The less pronounced difference of secreted HBsAg (Fig. 4D, upper) most likely is due to the nonlinear relationship of the ELISA and signal saturation.

PCR errors associated with *High Fidelity*^{plus} DNA polymerase and two approaches to overcome this problem. Besides the two clones described in Fig. 4, seven additional clones generated by the *High Fidelity*^{plus} DNA polymerase were tested by a transfection experiment as well. All nine clones were digested with BspQI and circularized by T4 DNA ligase prior to transfection. As is shown in Fig. 5A, about 40% of the ligated DNA corresponds to monomeric circles, and the remaining probably are dimers and trimers. Strikingly, clones 3 to 5 failed to replicate or replicated at extremely low levels (Fig. 5B, left). Clone 2 was defective at envelope protein expression and virion secretion (Fig. 5C and D), while clone 8 produced S protein of accelerated mobility and failed to secrete either HBsAg or HBeAg (Fig. 5D and F). Altogether, five out of the nine clones showed a defect in genome replication and/or protein expression. In contrast, all 10 clones generated by *Phusion* DNA polymerase under similar conditions (10 ng template DNA and 30 cycles of amplification) were capable of genome replication, virion secretion, and the expression of all of the viral proteins

examined (Fig. 5, right). Therefore, the replacement of *High Fidelity*^{plus} DNA polymerase with *Phusion* DNA polymerase will greatly alleviate the problem of PCR errors.

An alternative approach to overcome the complication of PCR errors is a population approach. Following the ligation of the HindIII-SacI-digested PCR product with similarly digested pUC18 vector (the problem of vector self ligation was minimized, as described in Materials and Methods), the whole transformation product was grown continuously in liquid culture followed by plasmid DNA extraction. Indeed, such PCR clone pools reproduced functional properties of the 4B genome relative to those of the 2A genome, such as higher replication capacity (Fig. 6A), more efficient virion secretion (Fig. 6B), lower HBeAg expression (Fig. 6E), less efficient core protein and HBeAg detection by the Dako antibody (Fig. 6D), and lower intracellular signals of the envelope proteins (Fig. 6C). These features are comparable to those observed with 2A and 4B clones (Fig. 3).

DISCUSSION

The circular nature of the HBV ccc DNA and the need to transcribe a terminally redundant pg RNA for genome replication pose problems for experiments using a single copy of cloned genome, which has its continuity disrupted by vector

FIG. 4. Functional characterization of two *High Fidelity*^{plus} PCR clones of the 4B genome. The two clones were transfected directly (lanes 1 and 4) following digestion with BspQI (lanes 2 and 5) or BspQI digestion plus treatment with T4 DNA ligase (lanes 3 and 6). The EcoRI dimer of clone 4B served as a positive control (lane 7). (A) On the left is a schematic representation of the PCR clone displaying positions of L, S, and core (C) promoters and coding sequences. On the right is a summary of protein expression and genome replication from the undigested monomer clone (M), monomer clone digested with BspQI (M+B), or monomer clone with further treatment with T4 DNA ligase (M+B+T). (B) Intracellular replicative DNA. (C) Intracellular L, M, and S envelope proteins and GAPDH. Please note that clone 4B produces extra size forms of the envelope proteins, such as gp30 for the S protein. (D) Secreted HBsAg and HBeAg, with values from 4B dimer set at 100% (based on three transfections).

sequence. Earlier workers in the field released the HBV genome from the vector by restriction enzyme digestion and converted it into a circle by ligation at a very low DNA concentration (13, 29). The subsequent adoption of tandem dimers as a functional substitute for the ccc DNA makes it unnecessary to manipulate the DNA for each transfection. The site-directed mutagenesis of such cloned HBV genomes has established the functional consequence of common HBV mutations, such as core promoter mutations and HBeAg-abolishing precore mutations (1, 2, 18, 31), although mutagenesis by restriction fragment exchange will convert a dimer back into a monomer to necessitate dimer construction for each mutant. Since the pg RNA is only about 1.1 times the genome length, an alternative approach is to clone 1.2 to 1.5 copies of the viral genome into a cloning vector, with 5' disposition of the core promoter-precore/core gene (11, 17, 27). A third approach is to clone the DNA version of the pg RNA (1.1× genome length) into a vector under a strong exogenous promoter, such as cytomegalovirus (CMV) or actin (7, 28), thus enabling robust pg RNA transcription and genome replication. Such high-replicating constructs are very useful in the screening of antiviral compounds and in analyzing the replication impact of

drug resistant mutations. However, they do not express HBeAg and cannot be used to compare the inherent replication capacities among clinical isolates.

To compare the biological properties among different HBV genotypes or to establish the functional properties of HBV isolates implicated in a particular clinical outcome, such as fulminant hepatitis, the direct functional analysis of the clinical isolates is needed. To this end, Günther and colleagues established a method for the amplification of full-length HBV genome from serum samples (12). The unique features of virion-associated HBV DNA make the precore region the only suitable primer binding site for the amplification of the full-length genome (Fig. 1). However, a dimer generated by BspQI-released HBV genome is not expected to replicate. Its further conversion into EcoRI dimer or SphI dimer is needed (Fig. 1). This complication, coupled with PCR errors and the presence of viral quasispecies in chronically infected individuals, necessitates the conversion of multiple PCR clones from a single patient into tandem dimers. This may explain why Günther and colleagues opted to use SspI-digested PCR product directly for transfection experiments. With such a population approach, the issue of PCR errors might be masked if all

FIG. 5. Genome replication, virion secretion, and protein expression of PCR clones derived from two thermophilic DNA polymerases. The 4B genome was amplified by 30 cycles of PCR using either *High Fidelity^{plus}* DNA polymerase or *Phusion* DNA polymerase and then cloned. The PCR clones were digested with BspQI and further treated with T4 DNA ligase before transfection to Huh7 cells. The uncut original EcoRI dimer of clone 4B served as a positive control for transfection. (A) A fraction of the purified DNA prior to transfection, with the first lane being HindIII-digested λ DNA. (D) Intracellular envelope proteins were detected by a monoclonal preS2 antibody (specific for the L and M proteins) and a polyclonal S antibody from Novus (less efficient at detecting L and M proteins) or just by the S antibody.

of the samples have similar viral titers and are amplified under identical PCR conditions (i.e., the same polymerase and same number of cycles of amplification).

In the present study, we optimized the full-length PCR transfection procedure in several ways. First, we demonstrated the high mutation rate of the *High Fidelity^{plus}* DNA polymerase. In fact, five out of nine PCR clones generated by 30 cycles of PCR showed a defect in genome replication, envelope protein/HBeAg expression, or virion secretion (Fig. 5). In contrast, all 10 PCR clones generated by *Phusion* DNA polymerase retained efficient genome replication, virion secretion, and protein expression. In this regard, *High Fidelity^{plus}* DNA polymerase has a 6 times lower mutation rate than *Taq* DNA

polymerase, whereas *Phusion* DNA polymerase has a 50 times lower mutation rate than *Taq*, indicating an accuracy eight times higher than that of *High Fidelity^{plus}* DNA polymerase. Furthermore, *Phusion* DNA polymerase is extremely processive and can work at much elevated temperatures. This will help melt secondary structures, increase specificity, and shorten the duration of PCR.

Second, we cloned the PCR product before transfection, which enables the unlimited supply of each HBV genome for repeat experiments or further mutagenesis. In addition to the isolation of individual clones, it is possible to generate a clone pool simply by growing the whole transformation product in liquid culture. Such a population approach will help study the

FIG. 6. Use of PCR clone pools to mask the effect of PCR errors on the functional characterization of the HBV genome. Both the 2A and 4B genomes were amplified by 30 cycles of PCR using *High Fidelity^{plus}* DNA polymerase. The PCR products were ligated to pUC18 vector, and ampicillin-resistant bacteria were grown directly in liquid culture. The resultant plasmid DNA was digested with BspQI, and the 3.2-kb HBV DNA was gel purified. The HBV DNA was either transfected directly to Huh7 cells (lanes 1 and 4) or treated with T4 DNA ligase prior to transfection (lanes 2 and 5). The EcoRI dimers of the original 2A and 4B clones were transfected in parallel to serve as controls (lanes 3 and 6). (A) Intracellular replicative DNA. (B) Virion DNA. (C) Intracellular envelope proteins and GAPDH. (D) Intracellular core protein and secreted HBeAg. (E) Secreted HBsAg and HBeAg from two experiments. Values from 2A dimer-transfected cells were set at 100%. The volume of supernatant used for the HBsAg assay was 15 μ l for 2A constructs and 70 μ l for 4B constructs. For HBeAg assay, 1 and 2 μ l were used, respectively.

cumulative properties of viral quasispecies, which is impossible with the dimer approach (each dimer has to be derived from a single HBV monomer clone). Third, the replacement of the expensive SapI enzyme (about \$1.00 per U) with BspQI, an isoschizomer at 1/10 the cost, will make the monomer approach more affordable. Fourth, we demonstrated that the level of replication originating from a linear HBV genome is very low. The circularization of the BspQI-linearized HBV genome with T4 DNA ligase greatly improves the replication and expression of core protein and HBeAg (Fig. 4B). Treatment with T4 DNA ligase also improved the DNA replication of HBV genome linearized at the ApaI, EcoRI, and SphI sites and enhanced the envelope protein expression of HBV genome linearized at the EcoRI site (Fig. 2 and 3). Certainly, the levels of genome replication and HBeAg expression achieved by circularized HBV genome still are lower than those of the EcoRI dimer, especially for clone 2A, which replicates at low levels (Fig. 6). Another drawback of this approach is the need to ligate DNA at a very large volume so as to minimize intermolecular ligation. We used phenol-chloroform extraction followed by ethanol precipitation to concentrate the DNA, because with Qiagen purification columns the minimum volume to elute DNA is 30 μ l, which makes DNA too diluted for transfection.

An interesting observation from the present study is the ability of undigested EcoRI monomer to express core protein and HBeAg and, more strikingly, the ability of undigested precore monomer to express much higher levels of envelope proteins than the digested form or the EcoRI dimer. The underlying mechanism remains unknown. The HBV genome

contains a single polyadenylation site (TATAAA; positions 1916 to 1921) located at the 5' end of the core gene. Since the pUC18 vector does not contain a polyadenylation signal, for the EcoRI monomer to express core protein and HBeAg would require the use of the HBV polyadenylation signal during the second pass of the transcript. This would predict an mRNA of 6.0 kb (3.3 kb for HBV and 2.7 kb for pUC18). Indeed, the production of terminally redundant pg RNA and precore RNA from the ccc DNA template also requires the suppression of the polyadenylation signal during the first pass of the transcript (26). The use of this polyadenylation signal for L and M/S protein expression from the undigested precore monomer predicts transcripts of 5.1 and 4.8 kb, respectively. Alternatively, a cryptic polyadenylation signal elsewhere in the HBV genome or in pUC18 vector is used. At any rate, the transfection of the uncut full-length PCR clones will permit the very efficient expression of viral envelope proteins. This increased sensitivity is useful to study immune escape mutants, which harbor amino acid substitutions in the S protein that diminish or abolish its detection by antibodies raised against wild-type protein (4, 6, 33). In addition, occult HBV infection also has been attributed to infection by such immune escape mutants (8, 16).

ACKNOWLEDGMENTS

This work was supported by the American Cancer Society grant RSG 06-059-01-MBC, NIH grants CA109733, CA133976, AA08169, and CA123544, Chinese Special Projects for Prevention and Control of Major Infectious Diseases (2008ZX10203, 2009ZX10603), and the Chinese High-Tech Program (2006AA02A411). Y.Q. is supported in

part by a predoctoral fellowship from the Chinese Ministry of Education.

REFERENCES

1. Baumert, T. F., S. A. Rogers, K. Hasegawa, and T. J. Liang. 1996. Two core promoter mutations identified in a hepatitis B virus strain associated with fulminant hepatitis result in enhanced viral replication. *J. Clin. Invest.* **98**:2268–2276.
2. Buckwold, V. E., Z. Xu, M. Chen, T. S. Yen, and J. H. Ou. 1996. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. *J. Virol.* **70**:5845–5851.
3. Carman, W. F., et al. 1989. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet* **ii**:588–591.
4. Carman, W. F., et al. 1990. Vaccine-induced escape mutant of hepatitis B virus. *Lancet* **336**:325–329.
5. Chu, C. J., and A. S. Lok. 2002. Clinical significance of hepatitis B virus genotypes. *Hepatology* **35**:1274–1276.
6. Cooreman, M. P., et al. 1999. Characterization of the reactivity pattern of murine monoclonal antibodies against wild-type hepatitis B surface antigen to G145R and other naturally occurring “a” loop escape mutations. *Hepatology* **30**:1287–1292.
7. Durantel, D., et al. 2004. A new strategy for studying in vitro the drug susceptibility of clinical isolates of human hepatitis B virus. *Hepatology* **40**:855–864.
8. El Chaar, M., D. Candotti, R. A. Crowther, and J. P. Allain. 2010. Impact of hepatitis B virus surface protein mutations on the diagnosis of occult hepatitis B virus infection. *Hepatology* **52**:1600–1610.
9. Garcia, T., et al. 2009. Drastic reduction in the production of subviral particles does not impair hepatitis B virus virion secretion. *J. Virol.* **83**:11152–11165.
10. Guarnieri, M., et al. 2006. Point mutations upstream of hepatitis B virus core gene affect DNA replication at the step of core protein expression. *J. Virol.* **80**:587–595.
11. Guidotti, L. G., B. Matzke, H. Schaller, and F. V. Chisari. 1995. High-level hepatitis B virus replication in transgenic mice. *J. Virol.* **69**:6158–6169.
12. Günther, S., et al. 1995. A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. *J. Virol.* **69**:5437–5444.
13. Hirschman, S. Z., P. Price, E. Garfinkel, J. Christman, and G. Acs. 1980. Expression of cloned hepatitis B virus DNA in human cell cultures. *Proc. Natl. Acad. Sci. U. S. A.* **77**:5507–5511.
14. Ito, K., K. H. Kim, A. S. Lok, and S. Tong. 2009. Characterization of genotype-specific carboxyl-terminal cleavage sites of hepatitis B virus e antigen precursor and identification of furin as the candidate enzyme. *J. Virol.* **83**:3507–3517.
15. Ito, K., et al. Impairment of hepatitis B virus virion secretion by single amino acid substitutions in the small envelope protein and rescue by a novel glycosylation site. *J. Virol.* **84**:12850–12861.
16. Jeantet, D., et al. 2004. Cloning and expression of surface antigens from occult chronic hepatitis B virus infections and their recognition by commercial detection assays. *J. Med. Virol.* **73**:508–515.
17. Khan, N., et al. 2004. Modulation of hepatitis B virus secretion by naturally occurring mutations in the S gene. *J. Virol.* **78**:3262–3270.
18. Li, J. S., et al. 1993. Hepatitis B virus genotype A rarely circulates as an HBe-minus mutant: possible contribution of a single nucleotide in the precore region. *J. Virol.* **67**:5402–5410.
19. Liang, T. J., K. Hasegawa, N. Rimon, J. R. Wands, and E. Ben-Porath. 1991. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. *N. Engl. J. Med.* **324**:1705–1709.
20. Magnius, L. O., and H. Norder. 1995. Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. *Intervirology* **38**:24–34.
21. Miyakawa, Y., and M. Mizokami. 2003. Classifying hepatitis B virus genotypes. *Intervirology* **46**:329–338.
22. Okamoto, H., et al. 1994. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. *J. Virol.* **68**:8102–8110.
23. Okamoto, H., et al. 1988. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J. Gen. Virol.* **69**:2575–2583.
24. Parekh, S., et al. 2003. Genome replication, virion secretion, and e antigen expression of naturally occurring hepatitis B virus core promoter mutants. *J. Virol.* **77**:6601–6612.
25. Raimondo, G., T. Pollicino, I. Cacciola, and G. Squadrito. 2007. Occult hepatitis B virus infection. *J. Hepatol.* **46**:160–170.
26. Russnak, R., and D. Ganem. 1990. Sequences 5' to the polyadenylation signal mediate differential poly(A) site use in hepatitis B viruses. *Genes Dev.* **4**:764–776.
27. Scaglioni, P. P., M. Melegari, and J. R. Wands. 1997. Posttranscriptional regulation of hepatitis B virus replication by the precore protein. *J. Virol.* **71**:345–353.
28. Seeger, C., B. Baldwin, and B. C. Tennant. 1989. Expression of infectious woodchuck hepatitis virus in murine and avian fibroblasts. *J. Virol.* **63**:4665–4669.
29. Sureau, C., J. L. Romet-Lemonne, J. I. Mullins, and M. Essex. 1986. Production of hepatitis B virus by a differentiated human hepatoma cell line after transfection with cloned circular HBV DNA. *Cell* **47**:37–47.
30. Tong, S. P., J. S. Li, L. Vitvitski, S. Benjelloun, and C. Trepo. 1991. Rapid screening for bacterial colonies harbouring tandem hepatitis B virus sequences by an oligonucleotide probe. *J. Virol. Methods* **32**:109–114.
31. Tong, S. P., J. S. Li, L. Vitvitski, and C. Trepo. 1992. Replication capacities of natural and artificial precore stop codon mutants of hepatitis B virus: relevance of pregenome encapsidation signal. *Virology* **191**:237–245.
32. Tsai, A., et al. 2009. Chimeric constructs between two hepatitis B virus genomes confirm transcriptional impact of core promoter mutations and reveal multiple effects of core gene mutations. *Virology* **387**:364–372.
33. Waters, J. A., et al. 1992. Loss of the common “A” determinant of hepatitis B surface antigen by a vaccine-induced escape mutant. *J. Clin. Invest.* **90**:2543–2547.
34. Zoulim, F., and S. Locarnini. 2009. Hepatitis B virus resistance to nucleos(t)ide analogues. *Gastroenterology* **137**:1593–1608.