Available online at www.sciencedirect.com
SCIENCE DIRECT®
Antiviral Research 61 (2004) 27–36
Antiviral Research
www.elsevier.com/locate/antiviral

In vitro antiviral susceptibility of full-length clinical hepatitis B virus isolates cloned with a novel expression vector

Huiling Yang, Christopher Westland, Shelly Xiong, William E. Delaney IV*

Gilead Sciences, 333 Lakeside Drive, Foster City, CA 94404, USA

Received 12 December 2002; accepted 21 July 2003

Abstract

Analyses of drug susceptibility and replication capacity for clinical HBV isolates have been hampered by the limitations of available in vitro culture systems. Site-directed mutagenesis has been used to study the effects of point mutations in recombinant laboratory HBV strains, however, the validity of such analyses are compromised since mutations are removed from their natural genetic context. Here we report the development of a new plasmid vector that facilitates the cloning and expression of full-length HBV genomes amplified from the sera of chronic hepatitis B patients. Using this vector, we cloned a total of 28 full-length HBV isolates from nine different patients. The majority of cloned HBV genomes (~70%) replicated in vitro and were suitable for further phenotypic characterization. Adefovir susceptibility was measured for clones from all nine patients. IC_{50} values were similar to those previously obtained with standard laboratory HBV strains and did not vary significantly between individual patient isolates (mean $IC_{50} = 0.24 \pm 0.08\,\mu M$). The vector described here enables the efficient phenotypic analysis of full-length HBV isolates from patients and will be useful in future studies including resistance surveillance, cross-resistance analyses, and novel drug-discovery.

Keywords: Hepatitis B virus; Clinical isolate; Drug susceptibility; Drug resistance; Adefovir

1. Introduction

In vitro drug susceptibility testing of clinical viral isolates plays important role in the management of HIV infection. With the availability of lamivudine ((−)-β-L-2′,3′-dideoxy-3′-thiacytidine), the recent approval of adefovir dipivoxil (9-[2-[bis-[(pivaloyloxy)methoxy][phosphinyl]methoxy]ethyl]-adenine), and the on-going clinical development of several other nucleoside analogs, phenotypic analysis of clinical HBV isolates is likely to play a similar role in the management of chronic hepatitis B. The in vitro analysis of clinical HBV isolates is currently difficult for several reasons. In contrast to HIV and many other viruses, cell culture systems that allow efficient in vitro infection and passaging of virus are not available for HBV. The HBV genome also has a complex circular arrangement that is organized into several overlapping reading frames (Seeger and Mason, 2000). Linearization of the HBV genome at any nucleotide results in the interruption of at least one open reading frame, which restricts strategies for directly generating functional HBV isolates by ligation into conventional cloning vectors.

Fortunately, the lack of in vitro infectivity can be overcome by transfecting plasmids encoding "greater-than-genome" length HBV sequences into liver cell lines. Following transfection, HBV pregenomic and messenger RNA are transcribed from the plasmid vector and progeny virus is replicated and released from cells. To date, the study of clinically-relevant HBV mutations has been largely limited to engineering point mutations into plasmids encoding laboratory strains of HBV. However, HBV is a heterogeneous virus that can be classified to eight distinct genotypes (Magnius and Norder, 1995; Stuyver et al., 2000; Arauz-Ruiz et al., 2002) and further subdivided based on the presence of a variety of clinically relevant mutations (e.g. precore, basal core promoter, HBIg/vaccine escape, and drug resistance mutations; for review see Burda et al., 2001; Baumert and Blum, 2000). The validity of analyses based on engineering point mutations into laboratory strains is compromised since mutations are removed from their natural genetic context and this may affect the overall phenotype of the virus. In addition, when multiple viral mutations are observed during longitudinal analyses, engineering individual mutations into a laboratory strain by site-directed mutagenesis becomes laborious. The development of efficient methods for cloning and expressing

* Corresponding author. Tel.: +1-650-522-5598; fax: +1-650-522-5890.
E-mail address: wdelaney@gilead.com (W.E. Delaney IV).

0166-3542/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2003.07.003

full-length clinical HBV isolates would greatly facilitate in vitro analyses of drug susceptibility and replication fitness.

Previously, Gunther et al. (1995) reported a primer set that enabled the PCR amplification of full-length HBV genomes. Furthermore, restriction of amplified genomes with the enzyme *Sap*I allowed removal of the flanking primer sequence and formation of circular HBV genomes suitable for transient transfection. In practice, however, it is difficult to enzymatically amplify sufficient viral DNA for comprehensive phenotypic studies. Cloning of amplified genomes by ligation into plasmid vectors would allow preparation of the cloned DNA on a large scale, however, due to the genetic organization of HBV, it is also difficult to directly clone amplified genomes in a way that permits efficient expression of the virus. The focus of the studies reported here was to develop more efficient methods for cloning and in vitro expression of patient-derived HBV isolates. We also investigated the natural variability in adefovir susceptibility of wild-type HBV isolates cloned from multiple patients.

2. Materials and methods

2.1. Patients and sera

Sera were collected from patients ($n = 9$) with either HBeAg-positive ($n = 5$) or HBeAg-negative ($n = 4$) chronic hepatitis B. All patients were HBsAg-positive, had serum HBV DNA levels of $\geq 5 \log_{10}$ copies/ml, and had elevated serum alanine amino-transferase levels (≥ 1.2 times the upper limit of normal). The Abbott AxSYM (Abbott Laboratories, Abbottt Park, IL) and the DiaSorin ETI-EBK (DiaSorin Inc., Stillwater, MN) ELISA assays were used to test for HBsAg and HBeAg, respectively. Serum HBV DNA was measured using the Amplicor^{TM} Monitor assay from Roche Diagnostics (Indianapolis, IN). Disease characteristics of these patients are presented in Table 1.

2.2. Extraction and amplification of HBV DNA

HBV DNA was purified from 200 μl aliquots of patient sera using the QIAamp Blood Kit (Qiagen, Chatsworth, CA). PCR amplification of full-length HBV genomes was performed using primers P1 (5′-CCG GAA AGC TTG AGC TCT TCT TTT TCA CCT CTG CCT AAT CA-3′) and P2 (5′-CCG GAA AGC TTG AGC TCT TCA AAA AGT TGC ATG GTG CTG G-3′) according to the methods of Gunther et al. (1995). If the initial PCR reactions were negative, a second round of PCR was performed under identical conditions using 5 μl of a 1:10 dilution of the first round reaction products as template.

Table 2
Primer sequences for generation of pHY106

Primer	Sequence (5′→3′)[a]
HY129	CCG GAC TCG A**GC ACC AGC ACC ATG CAA CT**T TTT GAA GAG CTC TTC T**TT TTC ACC TCT GCC TAA TCA**
HY130	GGA AAC AGC TAT GAC CAT G
HY131	CGT ATT GGG CGC TDT TCC GCT TCC TC
HY132	GAG GAA GCG GAA HAG CGC CCA ATA CG

[a] The bolded sequences indicate HBV DNA sequences; D = G+A+T; H = C + A + T.

2.3. Vector construction

The vector pHY106 contains a cytomegalovirus (CMV) promoter upstream of a short, recombinant HBV sequence that allows the in-frame insertion of a full-length HBV genome following *Sap*I digestion (Fig. 1D). pHY106 was derived from pHY92, a vector that encodes a replication competent 1.1× unit-length HBV genome (genotype A, serotype *adw*2, HBV strain identical to GenBank # AF305422) under the transcriptional control of the CMV promoter in the background of pBluescript KS(+) (Stratagene, La Jolla, CA) (Fig. 1A). The 1.1× unit-length HBV genome of pHY92 was excised using the restriction enzyme *Xho*I (Fig. 1B) and replaced with a short recombinant HBV fragment amplified from pHY92 with primers HY129 and HY130 (Table 2). The upstream primer HY129 contains 19 base-pair (nucleotides 1803–1821) and 20 base-pair (nucleotides 1822–1841) regions of the HBV genome linked by two *Sap*I sites (Fig. 1C). The downstream primer HY130 binds vector sequences and allowed amplification of a 160 base-pair HBV DNA fragment that includes the polyadenylation site at the 3′ end of the HBV genome (Fig. 1C). A *Sap*I site present in the parent vector was removed by site-directed mutagenesis with primers HY131 and HY132 (Table 2).

Table 1
Patient characteristics

	Patient number								
	1	2	3	4	5	6	7	8	9[a]
HBeAg status	Positive	Positive	Positive	Positive	Negative	Negative	Positive	Negative	Negative
Serum HBV DNA ($\log_{10}$ copies/ml)	6.00	8.33	7.80	6.38	6.17	7.52	7.16	7.17	7.95
HBV genotype	C	A	D	C	D	D	D	D	G
Location	France	Spain	Germany	Canada	Greece	France	Australia	Australia	Canada

[a] Patient had failed lamivudine therapy at the time of serum collection.

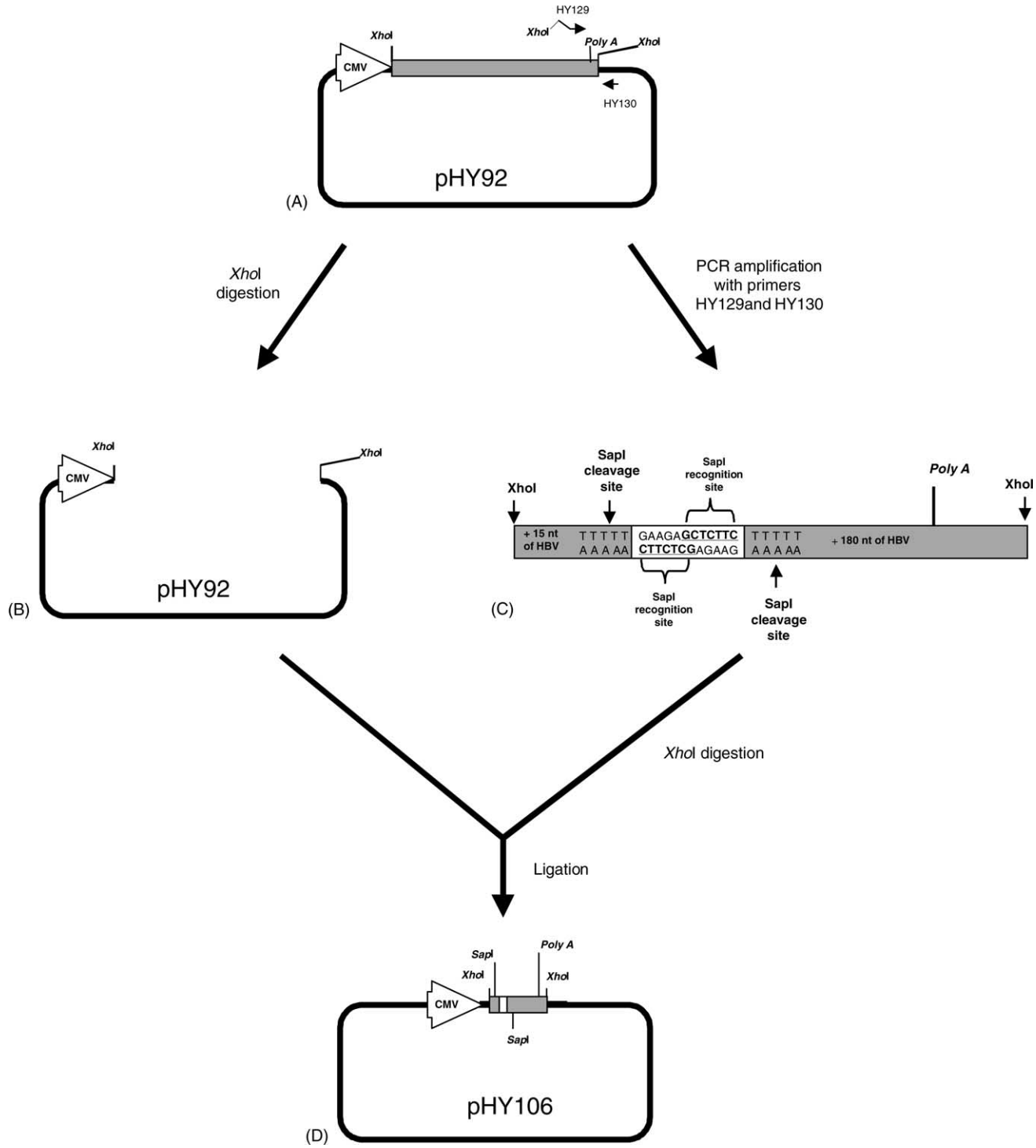

Fig. 1. Construction of pHY106. The vector pHY92 encodes a replication competent 1.1× unit-length HBV genome under the transcriptional control of a CMV promoter (A). Removal of the HBV genome from pHY92 via *Xho*I digestion (B) and PCR amplification of a small sequence from the 5′ end of the HBV genome with primers HY129 and HY130 (A, C) followed by ligation, produced pHY106, a vector that allows the insertion and expression of full-length HBV genomes (D) (also see Fig. 2).

2.4. Cloning of full-length HBV genomes

Following PCR amplification, HBV DNA was purified using a High Pure PCR Product Purification Kit (Roche Diagnostics). PCR products were then digested with the restriction enzyme *Sap*I (New England Biolabs, Beverly, MA) and ligated into *Sap*I-digested pHY106. Alternatively, the termini of amplified HBV genomic DNA were filled in by

incubation with Pfu DNA polymerase (Stratagene) at 72 °C for 30 min and HBV genomes were subsequently cloned into pCAP^s (Roche Diagnostics) at a Mlu Nl site by blunt-end ligation. Full-length HBV genomes cloned into pCAP^s could then be released by *Sap*I digestion and efficiently subcloned into pHY106 for use in transfection experiments.

2.5. In vitro replication of clinical HBV isolates

The ability of cloned HBV isolates to replicate in vitro was measured by transient transfection into HepG2 cells (ATCC, Manassas, VA). For the standard replication assay, six-well culture plates were seeded with 7.5×10^5 HepG2 cells/well. Sixteen hours post-seeding, cells were transfected with 4 μg of plasmid DNA using the Fugene 6 transfection reagent (Roche Diagnostics). Following transfection, cultures were fed fresh media and incubated for 72 h, after which intracellular HBV replicative intermediates were isolated as described (Delaney IV et al., 2001). Viral DNA was fractionated on 1% agarose gels and transferred to nylon membranes (Roche Diagnostics) using standard Southern blotting procedures (Sambrook et al., 1989). Membranes were hybridized to a ^{33}P-labeled HBV probe and viral DNA were quantified using a Storm 860 Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Transfection efficiency was not assayed during these experiments due to lack of HBeAg expression in many clones and significant differences in HBsAg sequence between the clones of different genotype. An analysis of isogenic HBV clones under identical conditions indicated that the coefficient of variation during Fugene-mediated transfection was 11.3% (Delaney IV et al., 2003).

2.6. Adefovir susceptibility testing

For standard antiviral assays, six-well culture plates were seeded with 7.5×10^5 HepG2 cells/well. Sixteen hours post-seeding, cells were transfected with 4 μg of plasmid DNA using the Fugene 6 transfection reagent. The next day, cells were fed fresh medium containing 0, 0.01, 0.1, 1, 10, or 25 μM adefovir (Gilead Sciences, Foster City, CA). Cells were treated with adefovir for one week, after which intracellular HBV replicative intermediates were isolated and quantified as described above. Regression analyses of antiviral data (based on the measurement of double-stranded HBV replicative intermediates) were performed with TableCurve2D software as previously described (Delaney IV et al., 2001; Colledge et al., 2000). Dose response equations were used to calculate the 50% inhibitory concentration (IC_{50}) of adefovir for each clinical isolate.

3. Results

3.1. Strategy and vector design

Since the PCR method of Gunther et al. amplifies linear full-length HBV genomes that lack both 5′ and 3′ sequences necessary for the expression of viral genes (Fig. 2A), a cassette vector was developed that contained the minimum HBV sequence necessary for viral transcription and replication after insertion of a genome-length PCR product. The vector pHY106 contains a CMV promoter, followed by an 18 nucleotide HBV sequence encoding the precore initiation site (plus the next two amino acids of the precore protein), a short heterologous linker sequence that contains two *Sap*I sites, and finally a 160 nucleotide region that encodes the carboxy terminus of the HBV X gene (five amino acids) as well as the polyadenylation signal for HBV mRNA. Following digestion of pHY106 and full-length HBV genomes with *Sap*I (Fig. 2B), HBV genomes can be inserted in-frame, resulting in the generation of replication-competent HBV clones that are 95% derived from patient virus (Fig. 2C). The CMV promoter upstream of the precore initiation site allows efficient transcription of the 3.5 kb pregenomic RNA following transfection of liver cell lines such as HepG2 or Huh-7 (Fallows and Goff, 1995; Ladner et al., 1997).

The minimal 5′ and 3′ sequences derived from the laboratory HBV strain included in pHY106 is highly conserved among published HBV isolates. Examination of 87 GenBank sequences (including multiple representatives of all HBV genotypes) revealed that the laboratory strain sequence would result in a single amino acid substitution in the precore reading frame of 2/87 sequences and a single amino acid substitution in the X gene of 1/87 sequences. Genotype G isolates encode a precore stop codon immediately following the methionine initiation site. While this stop codon would be replaced by a glutamine in the laboratory HBV sequence, all known isolates of genotype G contain a second precore stop codon downstream at position 28 (Kato et al., 2002a,b; Swenson et al., 2001; Vieth et al., 2002). Since all precore residues downstream of codon three will be derived from clinical HBV sequence, the cloning of a genotype G isolate into pHY160 would not restore the ability of genotype G virus to produce HBeAg in vitro. Thus, HBV isolates cloned into the pHY106 vector should be representative of patient virus with little or no genotypic changes contributed by the minimal laboratory strain sequence.

3.2. PCR amplification and cloning of full-length clinical HBV isolates

Using the method of Gunther et al., full-length HBV DNA was amplified from the majority of patient sera following a single round of PCR. For the remaining cases, a second round of PCR was used to successfully amplify HBV genomes. In general, it was more difficult to amplify full-length genomes from patients with lower levels of viremia (e.g. patients 1 and 5 who had viral loads of 6.00 and 6.17 $\log_{10}$ copies of HBV DNA/ml, respectively) (Table 1). Multiple full-length genomes were successfully cloned into the pHY106 vector either by direct cloning or by first subcloning into pCAP^s, a lethal selection vector. While more time consuming, the intermediate cloning step offered the

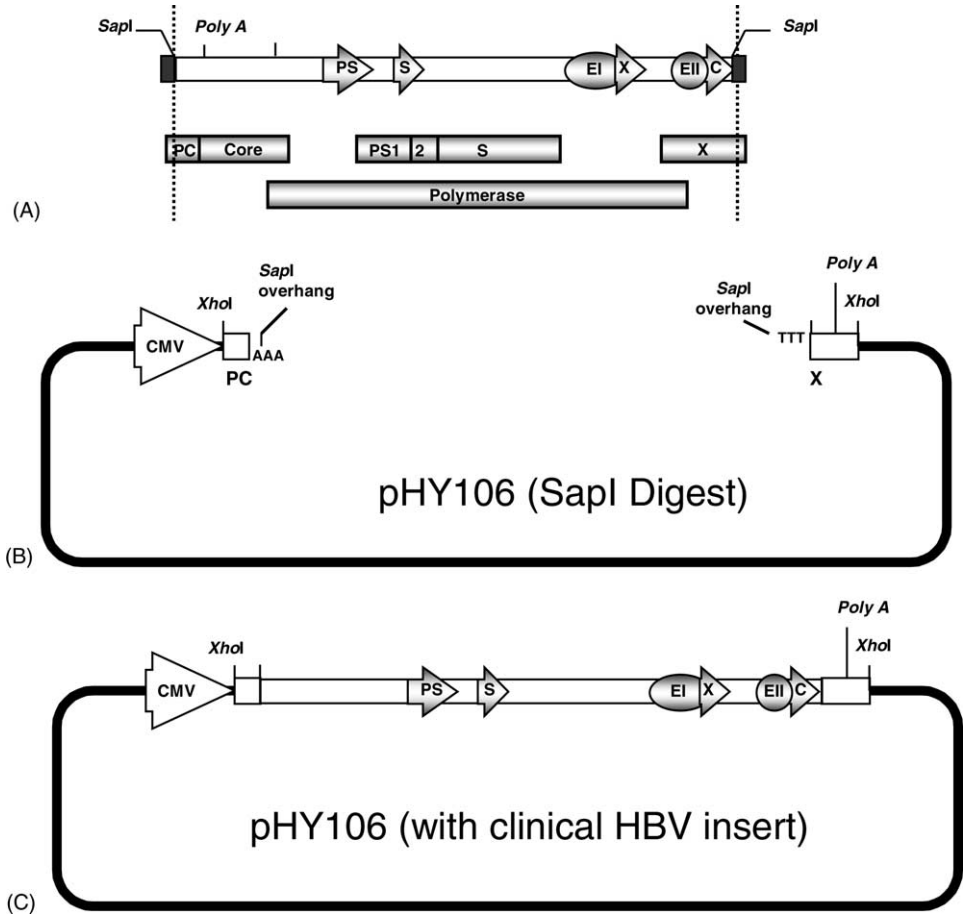

Fig. 2. Generation of replication competent clinical HBV isolates using pHY106. PCR by the methods of Gunther et al. yields linear genome-length HBV DNA that lacks 5′ and 3′ sequences necessary for HBV expression (primer sequence is indicated in black, patient HBV sequence is indicated in white) (A). HBV open reading frames (ORFs) for the precore (PC), core, preS1 (PS1), preS2 (2) surface (S), polymerase, and X genes are indicated by shaded blocks. Promoter sequences for the preS (PS), surface (S) and core (C) genes are indicated by shaded arrows; enhancer I (EI) and enhancer II (EII), elements are indicated by shaded ovals. Full-length HBV genomes can be digested with *Sap*I and inserted into *Sap*I-digested pHY106 (B) to produce a replication competent construct comprised of 95% patient-derived sequence (laboratory HBV strain sequence is indicated by solid gray boxes, patient-derived HBV sequence is indicated in white) (C).

following advantages: (1) efficient elimination of false positive clones (empty vectors) via lethal selection; (2) blunt end cloning into pCap^s allowed us to maintain patient isolates in a vector that affords convenient excision of the full-length clones via *Sap*I (cloning into the pHY106 vector destroys the *Sap*I sites and makes downstream molecular manipulations of the full-length patient clones more difficult). A total of 28 independent HBV isolates were obtained from the nine patient sera. Cloning was generally more successful (i.e. more independent clones could be obtained) from sera that produced a robust amplification of HBV DNA.

3.3. *In vitro replication of clinical HBV isolates*

All of the HBV isolates cloned into the pHY106 vector were analyzed for their ability to replicate in vitro. Cloned isolates were transfected into HepG2 cells and allowed to replicate for 72 h, after which intracellular replicative intermediates were extracted and analyzed by Southern blotting (Fig. 3). Interestingly, a marked variation in the levels of intracellular replication between individual clones was observed, even among clones derived from the same patient. Replication levels ranged from undetectable, to several-fold above a standard laboratory strain encoded by pHY92. The number of clones that yielded detectable replicative intermediates varied between patients, however the overall number of clones that replicated in vitro was 20/28 (71%). Importantly, replication competent clones suitable for further phenotypic analysis were isolated from all patients.

To investigate the genetic relatedness of HBV clones isolated from the same patients, we sequenced the entire reverse transcriptase domain of the 10 clones presented in Fig. 3. The percent identity between clones from the same patient as well as the average identity between individual clones and the consensus sequence (obtained by direct sequencing of PCR products) was then determined (Table 3). For patients 1 and 8, clones isolated from the same patient were highly homologous (>98% identity at both the nucleotide and amino

Table 3
Genotypic comparison of individual HBV clones

Patient	Number of clones	Nucleotide[a] identity between clones (%)	Average nucleotide identity to PCR consensus sequence[c] (%)	Amino acid[e] identity between clones (%)	Average amino acid[e] identity to PCR consensus sequence (%)
1	3	99.9–100[b]	99.9 ± 0.6[d]	99.7–100	99.9 ± 0.2[d]
7	5	95.5–98.8	98.2 ± 1.4	91.3–99.1	97.0 ± 3.1
8	2	98.9	99.5 ± 0.5	98.0	99.0 ± 1.0

[a] Nucleotide sequence is the reverse transcriptase domain of the HBV polymerase gene (1032 bases).
[b] Range of identity values.
[c] Population sequence obtained by direct sequencing of PCR products after amplification of the HBV reverse transcriptase gene from patient serum.
[d] Average ± S.D.
[e] Presumed amino acid sequence after translation with the Lasergene MegAlign program.

acid levels). This is consistent with the similar replication levels that different clones from these individual patients exhibited (Fig. 3). Patient 7 had more variability between individual clones with some clones showing 4.5 and 8.7% divergence at the nucleotide and amino acid levels, respectively. The greater genetic diversity identified in patient 7 clones was consistent with the observed variation in replication capacity (Fig. 3). Comparison of individual clones to the consensus sequence derived from the PCR products of each patient's sera revealed average identities of >98 and ≥97% at the nucleotide and amino acid levels, respectively (Table 3). This result confirms that population-based sequencing provides an accurate reflection of the individual quasi-species present in the serum of chronic hepatitis B patients.

3.4. Adefovir susceptibility of clinical isolates

A cell-based antiviral assay was used to test the adefovir susceptibility of a number of clinical isolates that replicated efficiently in cell culture (Fig. 4). Results from these assays indicated that all clinical HBV isolates had IC_{50} values similar to those previously reported for laboratory strains of HBV (Figs. 4 and 5). Mean IC_{50} values for individual clones ranged between 0.14 and 0.42 μM and the mean for all isolates was 0.24 ± 0.08 μM. The distribution of IC_{50} values for individual clones was log normal with all isolates falling between 0.6 and 1.8-fold of the mean. The pHY92 vector encoding the CMV-driven 1.1× unit-length laboratory HBV strain yielded an IC_{50} of 0.34 ± 0.10 μM during concurrent experiments.

3.5. Phenotypic confirmation of lamivudine resistance for an HBV isolate cloned from a patient that failed lamivudine therapy

One of the patients analyzed during these studies had clinical evidence of lamivudine failure (Table 1, patient 9). Three HBV isolates were cloned from this patient, and all had genotypic evidence of lamivudine resistance (rtL180M + rtM204V mutations). To further validate our cloning vector as a tool for phenotypic analysis, we tested the lamivudine susceptibility of one of these clones. As shown in Fig. 6A, the clone isolated from patient 9 had high level resistance to lamivudine (IC_{50} > 100 μM), but remained susceptible to adefovir (IC_{50} = 0.14 ± 0.06 μM) Figs. 5 and 6B).

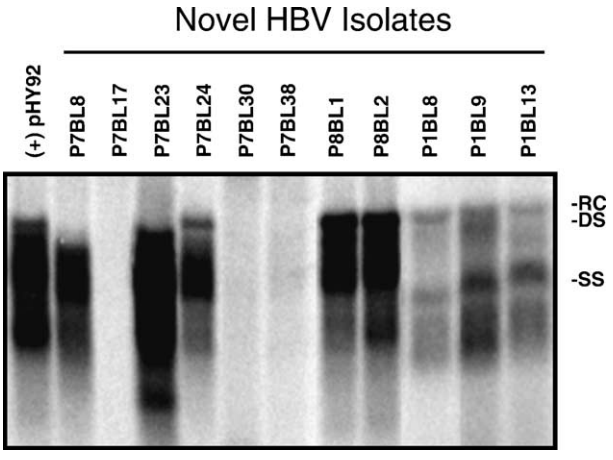

Fig. 3. Intracellular replication of clinical HBV isolates. HBV isolates were cloned into the vector pHY106 and transfected into HepG2 cells. 72 h post transfection, intracellular replicative intermediates were isolated and analyzed by Southern blotting. Relaxed circular (RC), double-stranded (DS), and single-stranded (SS) intermediates are indicated.

4. Discussion

The overall effect that a set of mutations will have on viral phenotype may differ significantly from that predicted from the effects of individual mutations. Combinations of lamivudine (rtL180M + rtM204V) and HBIg resistance mutations (sP120T and sG145R) produced an unexpected high replication phenotype in addition to resistance to both antiviral agents (Bock et al., 2002). More recently, the pre-core mutation G1896A has been shown to greatly enhance the replication capacity of HBV encoding the rtM204I (but not rtM204V) lamivudine resistance mutation (Chen et al., 2003). Thus, the effects of a single mutation are best studied in the proper genetic context so that potential interactions with co-existing mutations and polymorphisms are taken into account. The vector described here for cloning and expressing HBV isolates can be expected to produce more representative results than the alternative ap-

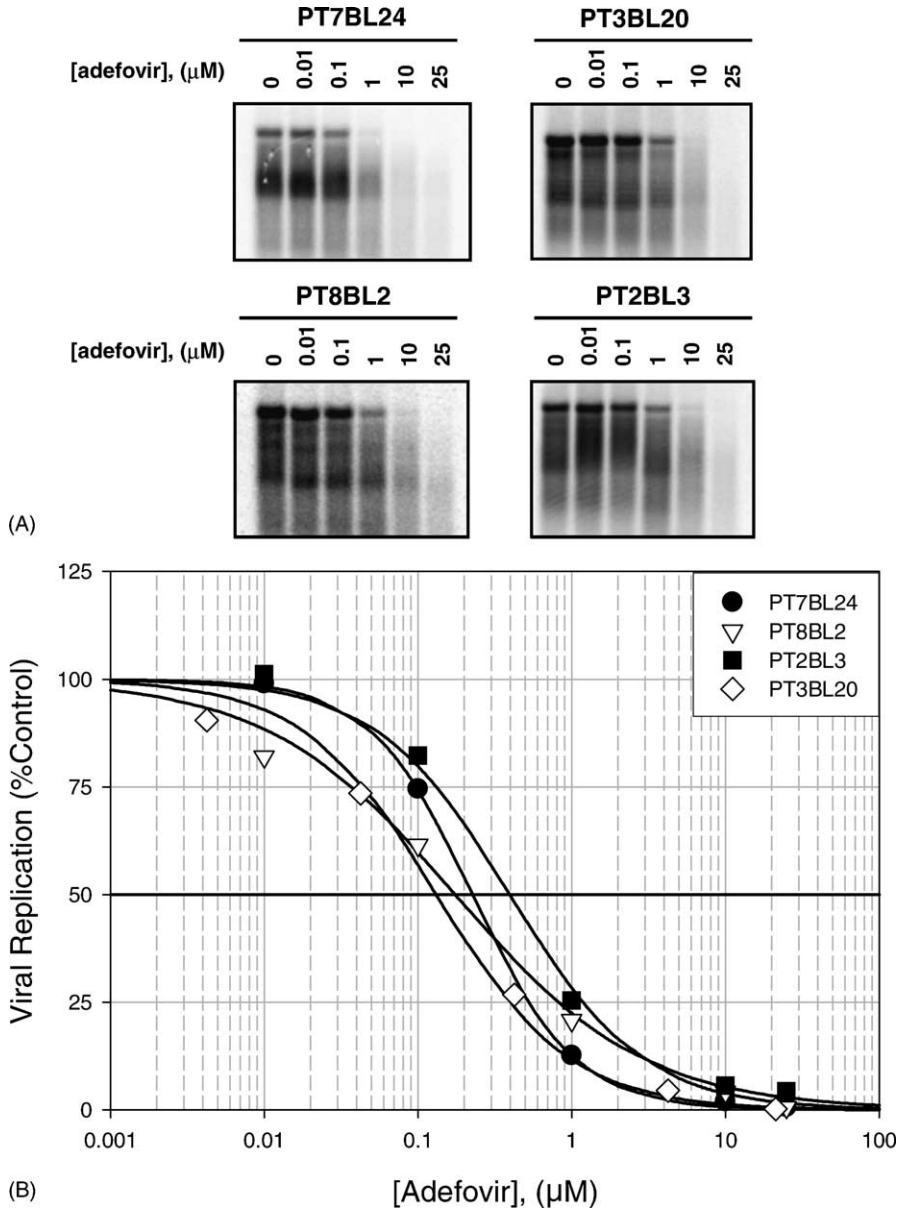

Fig. 4. IC_{50} determination for individual clinical isolates treated with adefovir. HepG2 cells were transfected with clinical HBV isolates cloned into the pHY106 vector. Transfected cells were treated with 0, 0.01, 0.1, 1, 10, or 25 μM adefovir for one week. At the end of the treatment period, intracellular replicative intermediates were isolated and analyzed by Southern blotting; representative blots for four isolates are shown (A). The inhibition of HBV replication by adefovir was quantified using a PhosphorImager and regression analyses were used to determine the IC_{50} of each isolate to adefovir (B).

proach of making single point mutations in laboratory HBV strains.

From our studies, it was apparent that there was significant variation in the in vitro replication of individual HBV isolates, even when the isolates were derived from the same patient. Approximately 70% of clones replicated to varying degrees, while the remainder did not yield detectable replication signals in vitro. Importantly, we were able to isolate replicating clones suitable for drug susceptibility testing from each of the nine patients we analyzed. There are several potential explanations for the variation in replication levels that we observed. These include: (1) HBV replicates as quasi-species in vivo and individual species are likely to vary with respect to replication capacity; (2) the replication environment in vitro is significantly different from that in vivo; this may differentially affect the in vitro replication of some viral species; (3) despite the use of a high fidelity PCR enzyme, PCR errors may occur and affect viral replication.

To investigate genetic variability among HBV isolates on an intra-patient level, we compared sequence identity in multiple clones isolated from three patients. This analysis revealed that individual clones isolated from a single patient could have substantial genetic differences (up to 8.7% divergence at the amino acid level). Since single, naturally occur-

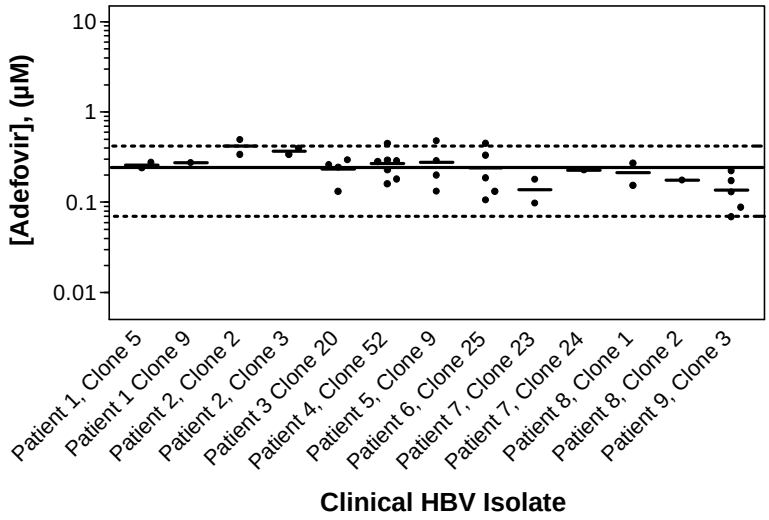

Fig. 5. Summary of adefovir susceptibility data for clinical HBV isolates. IC_{50} values for each clinical isolate were determined as described in Fig. 4. For each clone, the IC_{50} values from individual experiments are plotted as a single points and mean IC_{50} values are indicated by bars. The mean IC_{50} of all isolates is indicated by a solid line at 0.24 μM. Two standard deviations above and below the mean are indicated by dotted lines at lines at 0.40 and 0.08 μM, respectively.

ring amino acid changes in HBV polymerase (or other viral genes) are sufficient to profoundly change HBV replication levels in vitro (Lin et al., 2001; Suk et al., 2002), the degree of genetic variability that we observed provides a potential explanation for the phenotypic replication differences. Our observations are also consistent with those made by independent investigators who have assayed the in vitro replication levels of patient-derived HBV sequences (Jeantet et al., 2002; Kajiya et al., 2002).

During our studies, we did not note an obvious correlation between in vivo virological parameters (e.g. viral load, HBeAg status, HBV genotype) and the in vitro replication capacity of HBV clones. In fact, for many of the patients, the intra-patient variability in HBV isolate replication levels was as high as the inter-patient variability, making such an analysis difficult. While it possible that such a correlation could be established if more patients and/or HBV isolates were studied, it is also possible that patient differences, especially the degree of immune activity, may be the primary determinant of clinical parameters such as viral load.

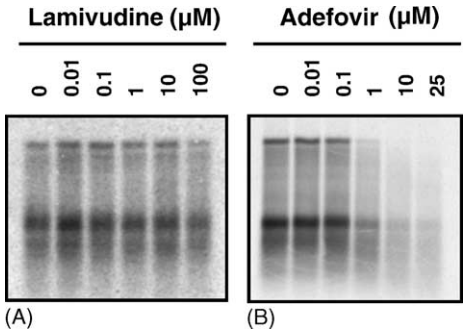

Fig. 6. Antiviral susceptibility of an HBV isolate cloned from a patient failing lamivudine therapy. An HBV isolate amplified from the serum of patient failing lamivudine therapy was cloned into the pHY106 expression vector and analyzed in vitro. Genotypic analysis revealed the presence of the rtL180M + rtM204V lamivudine resistance mutations. Phenotypic susceptibility testing to indicated that the isolate was resistant to lamivudine ($IC_{50} > 100$ μM) (A), but sensitive to adefovir ($IC_{50} = 0.14$ μM) (B).

There are several applications for the HBV expression methods described here. One major application will be resistance surveillance for antiviral agents, where it will be appropriate to compare the drug susceptibility of baseline and post-therapy isolates. Rapid analysis of patient HBV isolates will also be useful for determining the suitability of existing or novel antiviral agents or combinations of antiviral agents for treating patients with established drug resistance mutations. There are at least four major mutational patterns associated with lamivudine resistance (rtM204I, rtL180M + rtM204I, rtL180M + M204V, and rtV173L + rtL180M + M204V; Westland et al., 2001) as well as a partially overlapping set of famciclovir resistance mutations that include rtV173L, rtL180M, and rtV207I (Aye et al., 1997; Pichoud et al., 1999; Tillmann et al., 1999). Thus far, adefovir dipivoxil has demonstrated an excellent resistance profile, with no resistance mutations identified during the first year of phase III studies, or in smaller groups of patients treated for up to 136 weeks (Westland et al., 2003; Yang et al., 2001). Nevertheless, it is possible that viral resistance to adefovir will emerge in patients receiving long-term therapy and phenotypic analysis of patient HBV isolates will be necessary to confirm potential resistant genotypes. There are also a number of novel anti-HBV nucleoside drugs in clinical development. These include, emtricitabine (FTC), entecavir (BMS-200,475), telbivudine (L-dT), valtorcitabine (Val-L-dC), clevudine (L-FMAU), DAPD, and L-Fd4C (Delaney IV et al., 2002). Unlike adefovir, these drugs may have full or partial cross-resistance to lamivudine-resistant HBV as well as the potential to

select unique resistance mutations within HBV polymerase (Ladner et al., 1998; Fu et al., 1999; Ono-Nita et al., 2000; Delaney IV et al., 2001). Given the greater availability of anti-HBV agents and potential for cross-resistance, the clinical management of chronic hepatitis B virus may become similar to that of HIV where phenotypic testing of patient isolates contributes to the selection of effective individualized treatment regimens.

Our analysis of clinical isolates provides valuable data regarding potential natural variability in the susceptibility of wild-type HBV to adefovir. Although these analyses were limited to 13 novel isolates from nine patients, to our knowledge this is the largest panel of full-length HBV isolates that has been tested against adefovir or any other reverse transcriptase inhibitor. The IC_{50} values obtained for all clinical isolates fell within a relatively narrow concentration range of 0.14–0.42 μM adefovir (0.6–1.8 fold of the population mean) and were similar to what we and others have previously observed using genotype A or genotype D laboratory HBV strains (Yang et al., 2002; Delaney IV et al., 2001; Ono et al., 2001). Although more variability may be observed as the number of tested isolates increases, our observations thus far are consistent with in vitro drug susceptibility analyses of HIV with nucleoside and nucleotide analogs (Harrigan et al., 2001, 2002). Indeed, Harrigan and colleagues recently reported drug susceptibility data after testing the sensitivity of thousands of clinical HIV isolates to various antiretroviral drugs; these studies indicated that antiviral responses of HIV to nucleosides followed log normal distributions over with 97.5% of isolates falling within 3–4.5-fold of the population mean (Harrigan et al., 2001). In contrast, much more variability was observed in HIV susceptibility to non-nucleoside reverse transcriptase inhibitors, with some wild-type isolates displaying greater than 10-fold deviations from the population mean.

In conclusion, we have developed a novel plasmid vector that enables the efficient cloning and expression of HBV isolates from patient sera. Using this vector we were able to clone multiple HBV isolates from nine chronic hepatitis B patients. Despite differences in replication level and genetic background, adefovir demonstrated potent and similar activity against all isolates. The phenotyping methods described here will be useful for future studies including resistance surveillance, cross-resistance analyses, and novel drug discovery.

Acknowledgements

The authors thank Michael Miller and Craig Gibbs, for scientific discussion and critical review of this manuscript.

References

Arauz-Ruiz, P., Norder, H., Robertson, B.H., Magnius, L.O., 2002. J. Gen Virol. 83, 2059–2073.

Aye, T.T., Bartholomeusz, A., Shaw, T., Bowden, S., Breschkin, A., McMillan, J., Angus, P., Locarnini, S., 1997. J. Hepatol. 26, 1148–1153.

Baumert, T.F., Blum, H.E., 2000. Viral Hepat. Rev. 6, 177–192.

Bock, C.T., Tillmann, H.L., Torresi, J., Klempnauer, J., Locarnini, S., Manns, M.P., Trautwein, C., 2002. Gastroenterology 122, 264–273.

Burda, M.R., Gunther, S., Dandri, M., Will, H., Petersen, J., 2001. Antiviral Res. 52, 125–138.

Chen, R.Y., Edwards, R., Shaw, T., Colledge, D., Delaney IV, W.E., Isom, H., Bowden, S., Desmond, P., Locarnini, S.A., 2003. Hepatology 37, 27–35.

Colledge, D., Civitico, G., Locarnini, S., Shaw, T., 2000. Antimicrob. Agents Chemother. 44, 551–560.

Delaney IV, W.E., Edwards, R., Colledge, D., Shaw, T., Torresi, J., Miller, T.G., Isom, H.C., Bock, C.T., Manns, M.P., Trautwein, C., Locarnini, S., 2001. Antimicrob. Agents Chemother. 45, 1705–1713.

Delaney IV, W.E., Bartholomeusz, A., Locarnini, S.A., 2002. Expert Opin. Investig. Drugs 11, 169–187.

Delaney IV, W.E., Yang, H., Westland, C., Das, K., Arnold, E., Gibbs, C.S., Miller, M.D., Xiong, S., 2003. J. Virol., in press.

Fallows, D.A., Goff, S.P., 1995. J. Virol. 69, 3067–3073.

Fu, L., Liu, S.H., Cheng, Y.C., 1999. Biochem. Pharmacol. 57, 1351–1359.

Gunther, S., Li, B.C., Miska, S., Kruger, D.H., Meisel, H., Will, H., 1995. J. Virol. 69, 5437–5444.

Harrigan, P.R., Montaner, J.S., Wegner, S.A., Verbiest, W., Miller, V., Wood, R., Larder, B.A., 2001. AIDS 15, 1671–1677.

Harrigan, P.R., Miller, M.D., McKenna, P., Brumme, Z.L., Larder, B.A., 2002. Antimicrob. Agents Chemother. 46, 1067–1072.

Jeantet, D., Chemin, I., Mandrand, B., Zoulim, F., Trepo, C., Kay, A., 2002. Hepatology 35, 1215–1224.

Kajiya, Y., Hamasaki, K., Nakata, K., Nakagawa, Y., Miyazoe, S., Takeda, Y., Ohkubo, K., Ichikawa, T., Nakao, K., Kato, Y., Eguchi, K., 2002. J. Viral Hepat. 9, 149–156.

Kato, H., Orito, E., Gish, R.G., Bzowej, N., Newsom, M., Sugauchi, F., Suzuki, S., Ueda, R., Miyakawa, Y., Mizokami, M., 2002a. Hepatology 35, 922–929.

Kato, H., Orito, E., Gish, R.G., Sugauchi, F., Suzuki, S., Ueda, R., Miyakawa, Y., Mizokami, M., 2002b. J. Virol. 76, 6131–6137.

Ladner, S.K., Otto, M.J., Barker, C.S., Zaifert, K., Wang, G.H., Guo, J.T., Seeger, C., King, R.W., 1997. Antimicrob. Agents Chemother. 41, 1715–1720.

Ladner, S.K., Miller, T.J., Otto, M.J., King, R.W., 1998. Antivir. Chem. Chemother. 9, 65–72.

Lin, X., Yuan, Z.H., Wu, L., Ding, J.P., Wen, Y.M., 2001. J. Virol. 75, 11827–11833.

Magnius, L.O., Norder, H., 1995. Intervirology 38, 24–34.

Ono, S.K., Kato, N., Shiratori, Y., Kato, J., Goto, T., Schinazi, R.F., Carrilho, F.J., Omata, M., 2001. J. Clin. Invest. 107, 449–455.

Ono-Nita, S.K., Kato, N., Shiratori, Y., Yoshida, H., Kato, J., Goto, T., Schinazi, R.F., Carrilho, F.J., Omata, M., 2000. Hepatology 32, 393A.

Pichoud, C., Seigneres, B., Wang, Z., Trepo, C., Zoulim, F., 1999. Hepatology 29, 230–237.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. In: Ford, N., Nolan, C., Ferguson, M. (Eds.), Molecular Cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 9.1–9.62.

Seeger, C., Mason, W.S., 2000. Microbiol. Mol. Biol. Rev. 64, 51–68.

Stuyver, L., De Gendt, S., Van Geyt, C., Zoulim, F., Fried, M., Schinazi, R.F., Rossau, R., 2000. J. Gen. Virol. 81, 67–74.

Suk, F.M., Lin, M.H., Newman, M., Pan, S., Chen, S.H., Liu, J.D., Shih, C., 2002. J. Virol. 76, 12069–12077.

Swenson, P.D., Van Geyt, C., Alexander, E.R., Hagan, H., Freitag-Koontz, J.M., Wilson, S., Norder, H., Magnius, L.O., Stuyver, L., 2001. J. Med. Virol. 64, 305–311.

Tillmann, H.L., Trautwein, C., Bock, T., Boker, K.H., Jackel, E., Glowienka, M., Oldhafer, K., Bruns, I., Gauthier, J., Condreay, L.D., Raab, H.R., Manns, M.P., 1999. Hepatology 30, 244–256.

Vieth, S., Manegold, C., Drosten, C., Nippraschk, T., Gunther, S., 2002. Virus Genes 24, 153–156.

Westland, C.E., Yang, H., Namini, H., Gibbs, C.S., Miller, M.D., Fry, J., Brosgart, C.L., Xiong, S., 2001. Comparison of Anti-HBV Activity of Adefovir Against Different Lamivudine-Resistant HBV Strains in Vitro and in Liver Transplant Patients. Hepatology 34, 446A.

Westland, C., Yang, H., Delaney IV, W.E., Gibbs, C., Miller, M., Wulfsohn, M., Fry, J., Brosgart, C., Xiong, S., 2003. Hepatology 38, 96–103.

Yang, H., Westland, C., Delaney IV, W.E., Ho, V., Miller, M.D., Gibbs, C.S., Fry, J., Brosgart, C.L., Xiong, S., 2001. Hepatology 34, 316A.

Yang, H., Westland, C.E., Delaney IV, W.E., Heathcote, E.J., Ho, V., Fry, J., Brosgart, C., Gibbs, C.S., Miller, M.D., Xiong, S., 2002. Hepatology 36, 464–473.