Hepatitis B virus (HBV)-specific short hairpin RNA is capable of reducing the formation of HBV covalently closed circular (CCC) DNA but has no effect on established CCC DNA in vitro

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Hepatitis B virus (HBV) covalently closed circular (CCC) DNA is the source of HBV transcripts and persistence in chronically infected patients. The novel aspect of this study was to determine the effect of RNA interference (RNAi) on HBV CCC DNA when administered prior to establishment of HBV replication or during chronic HBV infection. HBV replication was initiated in HepG2 cells by transduction with HBV baculovirus. Subculture of HBV-expressing HepG2 cells at 10 days post-transduction generates a system in which HBV replication is ongoing and HBV is expressed largely from CCC DNA, thus simulating chronic HBV infection. HepG2 cells were transduced with short hairpin RNA (shRNA)-expressing baculovirus prior to initiation of HBV replication or during chronic HBV replication, and the levels of HBV RNA, HBV surface antigens (HBsAg) and replicative intermediates (RI), extracellular (EC) and CCC DNA species were measured. HBsAg, HBV RNA and DNA levels were markedly reduced until day 8 whether cells were transduced with shRNA prior to or during a chronic infection; however, the CCC DNA species were only affected when shRNA was administered prior to initiation of infection. We conclude that RNAi may have a therapeutic value for controlling HBV replication at the level of RI and EC DNA and for reducing establishment of CCC DNA during HBV infection. Our data support previous findings demonstrating the stability of HBV CCC DNA following antiviral therapy. This study also reports the development of a novel HBV baculovirus subculture system that can be used to evaluate antiviral effects on chronic HBV replication.

INTRODUCTION

More than 350 million individuals worldwide are chronically infected with hepatitis B virus (HBV) (Kane, 1995; Margolis, 1998) and they are 100 times more likely to develop hepatocellular carcinoma than uninfected individuals (Beasley, 1988). Current treatments for HBV include nucleoside analogues and alpha interferon (IFN-α) administration. IFN-α has a low population response rate (~30%) and adverse side effects (Ganem & Prince, 2004). Nucleoside analogues directly block viral replication by targeting the reverse transcriptase, but do not affect viral antigen load (Lau et al., 2000; Papatheodoridis et al., 2002). This limitation can induce a host immune response resulting in hepatocellular injury (Ganem & Prince, 2004). Another major limitation of nucleoside analogues is the development of drug-resistant mutants (Melegari et al., 1998). Due to low efficacy and undesirable outcomes of these treatments, new therapies capable of targeting multiple aspects of the HBV viral life cycle are needed.

The HBV genome contains four overlapping open reading frames that encode the core, polymerase, surface and X proteins, as well as the pre-genomic RNA (pgRNA), the template for HBV genome replication (Ganem & Varmus, 1987). The unique arrangement of the HBV transcripts and the fact that replication occurs through an RNA intermediate make HBV an attractive target for RNA interference (RNAi) therapy.

RNAi is the process by which gene expression is silenced through the sequence-specific degradation of mRNA (Fire et al., 1998; Lau et al., 2001). RNAi is mediated by activation of the RNA-induced silencing complex through its association with 21–23 nt small interfering RNAs (siRNA) derived from Dicer-processed long double-stranded RNAs (Hammond et al., 2000, 2001). Several studies have
demonstrated the effectiveness of synthetic siRNA, vector-generated siRNA and short hairpin RNA (shRNA) in the inhibition of HBV replication and antigen production (Chen et al., 2007; Jia et al., 2006; McCaffrey et al., 2003; Moore et al., 2005; Shlomai & Shaul, 2003; Uprichard et al., 2005).

Retroviral vectors expressing shRNA sequences are capable of efficiently transducing hepatocytes and inducing long-term inhibition of HBV replication (Moore et al., 2005); however, successful treatment using retroviral vectors is dependent upon cell division and genomic integration. The baculovirus Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) can mediate gene delivery to a wide variety of mammalian cells (reviewed by Kost & Condreay, 2002; Kost et al., 2005). Recombinant baculoviruses are capable of delivering target genes to liver-derived cells with efficiencies greater than 90 % (Boyce & Bucher, 1996; Delaney & Isom, 1998; Hofmann et al., 1995). We previously reported the generation of a system for studying HBV replication in vitro, in which an HBV-expressing baculovirus efficiently delivers the HBV genome to HepG2 cells to initiate a productive infection wherein HBV transcripts, intracellular and secreted HBV antigens, HBV DNA replicative intermediates (RI) and HBV covalently closed circular (CCC) DNA are produced and extracellular (EC) HBV virions are secreted (Abdelhamed et al., 2002, 2003; Delaney & Isom, 1998; Heipertz et al., 2007). In this system, HBV transcription is initially driven from input HBV baculovirus DNA; however, during the first few days post-transduction, the input baculovirus DNA declines to levels virtually undetectable by Southern blot analysis and HBV CCC DNA begins to accumulate inside the nucleus where it drives HBV transcription for at least 4 weeks post-transduction (Abdelhamed et al., 2002; Heipertz et al., 2007).

Recent studies demonstrated that a baculovirus construct expressing shRNAs targeting lamin A/C can be used to transduce HepG2 cells, resulting in knockdown of the target gene (Nicholson et al., 2005). This suggests that recombinant baculovirus is an efficient vector for delivery of shRNA to HepG2 cells. In this study, we generated a baculovirus expressing an HBV-specific shRNA and tested its ability to inhibit HBV replication when delivered prior to the initiation of HBV replication established by the previously described HBV baculovirus system (Delaney & Isom, 1998). This study also reports the development of a novel subculture system, derived from the original HBV baculovirus system, that mimics a chronic HBV infection with HBV transcription driven from HBV CCC DNA, the primary source of HBV transcripts and causative agent of HBV persistence in patients. The subculture system was used to test the ability of the anti-HBV shRNA-expressing baculovirus to inhibit chronic HBV replication.

METHODS

Cell culture. HepG2 cells were cultured in minimal essential medium (MEM; Invitrogen) supplemented with 10 % heat-inactivated fetal bovine serum (FBS; HyClone) (MEM-FBS) and incubated at 37 °C in a humidified incubator at 5 % CO2 (Knowles et al., 1980). SF21 insect cells were cultured at 28 °C without CO2 in a non-humidified incubator and maintained in Grace’s insect medium supplemented with yeastolate (Mediatech), lactalbumin hydroxide (Mediatech) and 10 % FBS.

Generation of shRNA sequences and expression vectors. Potential shRNA sequences against various regions of the HBV genome were generated using Ambion’s Target Finder (www.ambion.com). pSilencer plasmids (pSiU6S, pSiU6E1, pSiU6E2X and pSiU6C) were generated using the oligonucleotides given in Supplementary Table S1 (available in JGV Online). Oligonucleotides were annealed and ligated into pSilencer 2.0-U6 BamHI and EcoRI sites according to the manufacturer’s instructions (Ambion). pSilencer 2.0-U6 negative control (Ambion) was used as a negative control (pSiU6Control).

DNA sequencing was used to verify all pSiU6 plasmids created. pSiU6 plasmids were digested with HindIII and EcoRI and the U6 promoter–shRNA sequence was subsequently cloned into the HindIII–EcoRI sites of pBlueBack.5 (pBB4.5; Invitrogen) to generate pBBU6E1, pBBU6E2X, pBBU6C, pBBU6S and pBBU6Control.

Generation of HBV shRNA-expressing baculovirus. HBV baculovirus was produced as described previously (Delaney & Isom, 1998). This method was used to generate shRNA-expressing baculovirus (Delaney & Isom, 1998). Briefly, pBBU6S and pBBU6Control were individually co-transfected along with linear AcMNPV baculovirus DNA into SF21 cells using the BacNBlue transfection kit (Invitrogen), and baculoviruses were amplified in SF21 cells from clones isolated by plaque assay according to the manufacturer’s instructions. DNA from baculoviruses was extracted as described by O’Reilly (1997), subjected to HindIII and EcoRI restriction enzyme digestion and visualized by Southern blotting according to methods described by Sambrook et al. (1989) to determine which virus isolates contained intact U6–shRNA inserts. Positive baculoviruses were amplified in SF21 suspension cultures, concentrated and titrated by end-point dilution (O’Reilly, 1997).

Co-transfection and baculovirus transduction. pBBU6S, pBBU6E1, pBBU6E2X, pBBU6C and pBBU6Control (1.5 µg) were individually co-transfected along with 1.5 µg pBB4.5HBV1.3 into HepG2 cells 16–24 h after seeding in 60 mm plates using Effectene transfection reagent (Qiagen) according to manufacturer’s instructions. pBB4.5HBV1.3 (1.5 µg), transfected into HepG2 cells as described above, served as a negative control. Cells were fed every other day and harvested 4 days post-transfection for analysis of HBV RI. All transfections were carried out in duplicate and repeated in triplicate in independent experiments.

Baculovirus transduction of HepG2 cells was carried out as described previously (Abdelhamed et al., 2003; Delaney & Isom, 1998). Briefly, HepG2 cells were seeded at a confluency of 20–40 % in 60 mm plates, 16–24 h prior to baculovirus transduction. On the day of transduction, a mean cell count was determined. The appropriate volume of high-titre baculovirus stock needed to obtain the desired m.o.i. was calculated and diluted in 0.5 ml MEM-FBS. Baculovirus inoculum was applied drop-wise to HepG2 cells, allowed to absorb for 1 h at 37 °C with gentle rocking every 15 min and removed by gently washing cells twice with PBS. HepG2 cells were re-fed and maintained as described above.

HBV DNA extraction and Southern blot. Intracellular HBV RI and EC DNA were extracted from HepG2 cells as described previously (Abdelhamed et al., 2002, 2003). HBV RI and EC DNA were separated in 1 % agarose gels and Southern blotted (Sambrook et al., 1989). Membranes were hybridized with probe generated from a full-length double-stranded HBV genome radiolabelled with [α-32P]dCTP using a random prime labelling kit (Roche Diagnostics).
To isolate CCC DNA, HBV-infected HepG2 cells were harvested and pelleted by centrifugation (1000 g for 5 min) in cold PBS. Pellets were lysed on ice for 20 min by the addition of 750 µl cold PBS containing 0.005% Nonidet P-40 (Roche). The cytoplasmic fraction was separated from the nuclear fraction by centrifugation at 1700 g at 4 °C. HBV RI DNA was extracted from the cytoplasmic fraction as described previously (Abdelhamed et al., 2002, 2003). The nuclear pellet was resuspended in 400 µl Tris/EDTA (TE) buffer (10mM Tris pH 8.0/1 mM EDTA pH 8.0: 50:1) and lysed using a modified, previously described procedure (Zhang & Summers, 2000). Briefly, 300 µl of a solution containing 0.15 M NaOH and 6% SDS was added to the nuclear pellet, then rotated for 10 min at room temperature, followed by incubation at 37 °C for 10 min to allow for the irreversible denaturation of cellular DNA. The alkaline lysate was neutralized with 200 µl 3 M potassium acetate neutralization buffer (pH 5) and centrifuged at 21000 g for 20 min at 4 °C. The supernatant was phenol and chloroform extracted, and nucleic acid was recovered by 2-propanol precipitation with the addition of 10 µg tRNA. Precipitated nucleic acid was resuspended in TE buffer (10 mM Tris/1 mM EDTA) and phenol and chloroform extracted, and nucleic acid was recovered by 2-propanol precipitation with the addition of 10 µg RNase ml⁻¹ for 30 min at 37 °C and analysed by electrophoresis and Southern blot as described above.

**HBV RNA extraction and Northern blot.** The single-step acid guanidinium method (Chomczynski & Sacchi, 1987) was utilized to extract total RNA from HepG2 cells. Total RNA (10 µg) was analysed by electrophoresis and Northern blotting. The membranes were hybridized with HBV and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific [α-32P]dCTP-radiolabelled probes.

**Baculovirus DNA extraction and Southern blot.** Total DNA was harvested from baculovirus-transduced HepG2 cells at various time points post-transduction as described previously (Abdelhamed et al., 2002). Samples were analysed by electrophoresis and Southern blotting. An [α-32P]dCTP-radiolabelled 332 bp fragment from pBB4.5 was used to specifically detect baculovirus DNA.

**Subculture of HBV-infected HepG2 cells.** HepG2 cells were infected with HBV-expressing baculovirus (m.o.i. of 100) according to methods described previously (Abdelhamed et al., 2003; Delaney & Isom, 1998). On day 10 after baculovirus-mediated HBV infection, HBV-infected HepG2 cells were subcultured 1:4 into 60 mm dishes.

**Measurement of secreted HBV surface antigen (HBsAg).** Secreted HBsAg was measured from culture media collected 24 h after refedding by ELISA using Auszyme Monoclonal kit (Abbott Laboratories) according to the manufacturer’s instructions. Appropriate dilutions of culture media were made as necessary.

**Data analysis and statistical significance.** Northern and Southern blots were visualized with a PhosphorImager; RNA and DNA bands were quantified using ImageQuant (Molecular Dynamics). Significance was determined using a homoscedastic two-tailed Student’s t test. P values ≤ 0.05 were considered significant.

**RESULTS**

**Selection and screen of shRNA sequences**

Various regions of the HBV genome were chosen as potential RNAi target areas and four shRNA sequences against these regions were generated using Ambion’s Target Finder. All four shRNA sequences target the HBV 3.5 kb RNA (Fig. 1a), which is the template for HBV genomic replication and also the mRNA for the HBV polymerase, HBV e antigen (HBeAg) and core protein. shRNAs U6S and U6E1 also target the 2.4 and 2.1 kb mRNA transcripts for HBsAg. U6E2X targets all HBV transcripts, including the HBV X antigen mRNA.

To test the ability of each shRNA sequence to inhibit HBV replication in cell culture, a co-transfection assay was conducted. All four shRNA sequences were cloned into the baculovirus transfer vector pBB4.5. HepG2 cells were co-transfected with pBB4.5HBV1.3, a greater than genome length HBV expression plasmid (Delaney & Isom, 1998), along with either pBBU6S, pBBU6E1, pBBU6E2X, pBBU6C, pBBU6Control or media alone (mock). Four days after co-transfection, cells were harvested and HBV RI was extracted and analysed by Southern blot (Fig. 1b). Of the four shRNA sequences tested, only U6S was capable of markedly inhibiting HBV RI levels. To our knowledge, the effects of U6E1 and U6E2X on HBV replication have not been evaluated. Previous studies showed that the U6C sequence caused a marked reduction in both HBV mRNA levels as well as HBsAg and HBeAg levels (Zhang et al., 2004). We did not investigate the effect of U6C on HBV mRNA or antigen expression, making it difficult to compare the two studies. In addition, different HBV systems and transfection methods were used, which may...
account for the differences in effectiveness of the sequence. Although there is no known explanation for the failure of the other anti-HBV shRNA sequences tested, it is possible that there may be an inherent flaw in their design that affects proper strand incorporation or other biochemical factors necessary for function. U6S targets a specific region in the HBsAg reading frame that is conserved throughout all HBV genotypes and may contribute to its potency. The U6S sequence was selected for future experiments.

Pretreatment with shRNA-expressing baculovirus

shRNA delivery is a major obstacle and determinant of RNAi efficacy. To overcome these issues, baculoviruses expressing U6S and U6Control were generated and their ability to prevent and inhibit HBV replication was examined. HepG2 cells were transduced with U6S or U6Control baculoviruses at an m.o.i. of 100. HBV replication was initiated 24 h later, by transducing the cells with HBV baculovirus (m.o.i. of 100). Representative Southern blots for RI (Fig. 2b), EC (Fig. 2d) and CCC (Fig. 2f) DNA from mock-, U6Control- and U6S-treated HepG2 cells transduced with HBV baculovirus are shown. It is important to note that, at least with regard to single-stranded HBV DNA, when HepG2 cells are transduced with HBV baculovirus, RI is detectable by day 1, EC is not detectable until day 2 and CCC DNA is not detectable until day 4 post-transduction (Abdelhamed et al., 2002; Delaney & Isom, 1998; Heipertz et al., 2007). For this reason, quantitative data are provided beginning with day 1 for RI, day 2 for EC and day 4 for CCC DNA. In these and other experiments (e.g. Fig. 5f), more than one band was sometimes observed at the expected CCC DNA position. We do not know the underlying reason for this; however, both bands were converted into a single 3.2 kb band upon treatment with the single-cut enzyme XhoI (data not shown). Therefore, both bands were considered as CCC DNA and their sum was used to calculate the relative amount of CCC DNA in the samples. U6Control treatment caused some inhibition of HBV replication compared with mock-treated cells; therefore, the effects of U6S on HBV replication, calculated using data from three independent experiments, are presented as a normalized mean relative to U6Control-treated cells. In the U6S-treated lanes of Fig. 3(a), there appears to be an RNA species migrating between the normal detection positions of the 2.4 and 2.1 kb bands. Although we have not characterized this band, it was commonly detected in U6S-treated samples and may be the product of differential splicing.

The effect of U6S on levels of HBsAg secreted into the culture media was determined. Results were calculated using data from three independent experiments and are presented as a normalized mean relative to U6Control-treated cells. U6S-expressing baculovirus significantly inhibited HBsAg levels compared with U6Control-treated cells (Table 1).

HBV baculovirus/HepG2 subculture system

Long-term cell culture models of HBV chronic infection are limited and many rely on cell lines with integrated copies of the HBV genome. To test the ability of shRNA U6S-expressing baculovirus to inhibit HBV replication under chronic conditions, we developed a novel system where HBV transcription occurs predominantly from CCC DNA. HepG2 cells were transduced with HBV baculovirus (m.o.i. of 100). At day 10 post-transduction, a time at which input baculovirus DNA is markedly reduced (determined by Southern blot analysis) and HBV transcription occurs from nuclear HBV CCC DNA (Heipertz et al., 2007), the HBV-expressing HepG2 cells were subcultured at a ratio of 1:4. The subcultured cells continued to produce high levels of HBV RI (Fig. 4a), EC (Fig. 4b) and CCC (Fig. 4c) DNA that was easily detectable by Southern blot analysis from day 1 until day 10 post-subculture. Southern blots for HBV RI, EC and CCC DNAs from unsplit cells at day 10 post-transduction are not shown; however, data from PhosphorImager scans of these blots were used to calculate the relative levels of HBV DNA in subcultured cells compared to day 10 unsplit cells in Fig. 4(d).

To determine the levels of baculovirus DNA in the subculture system, HepG2 cells were transduced with HBV baculovirus (m.o.i. of 100) and total DNA was extracted at various time points after transduction, from both unsplit and subcultured cells. Baculovirus DNA levels markedly decreased until day 5 post-transduction and continued to decrease, reaching virtually undetectable levels by day 10 post-transduction (Fig. 4e, f). Baculovirus DNA levels were essentially undetectable at day 21 post-transduction and day 10 post-subculture. These results agree with and extend previously published data (Heipertz et al., 2007).
shRNA-mediated inhibition of a chronic HBV infection in vitro

We used the baculovirus subculture system to investigate the effect of U6S-expressing baculovirus on HBV replication during a chronic infection. HepG2 cells were subcultured 1:4 10 days post-transduction with HBV baculovirus. The infected cells were transduced with U6S or U6Control baculovirus (m.o.i. of 100) 24 h post-subculture and the inhibitory effect of U6S on HBV replication was examined. One of the strengths of the subculture system is that, at 24 h after plating, the HBV-expressing HepG2 cells are at an appropriate cell density.
for transduction by a recombinant baculovirus, in this case, baculoviruses expressing U6S or U6Control. The inhibition of HBV replication by U6S was calculated using data from three independent experiments and is presented as a normalized mean relative to U6Control-treated cells (Fig. 5a, c, e). Eight days after U6S transduction, U6S significantly inhibited HBV RI DNA levels by 68.4 ± 5.2 % (P=3.81 E−07) and EC DNA levels by 63.7 ± 7.7 % (P=4.42 E−05) compared with cells transduced with U6Control (Fig. 5a, c, respectively). No changes in HBV CCC DNA levels were observed when cells were transduced with U6S baculovirus (Fig. 5e, f).

Northern blot analysis was conducted to determine whether the observed inhibition in HBV RI and EC DNA levels was a consequence of U6S-mediated degradation of HBV transcripts. U6S was capable of reducing the HBV 2.4/2.1 kb and 3.5 kb transcripts (Fig. 6a). Results were normalized to GAPDH levels and the effect of U6S on HBV mRNA levels was calculated from three independent experiments and presented as a normalized mean relative to U6Control-treated cells (Fig. 6b). U6S significantly decreased total HBV mRNA levels by 81.1 ± 2.3 % (P=6.90 E−05) 4 days after U6S transduction compared with U6Control-treated cells (Fig. 6b).

Table 1. Inhibition of HBsAg secretion by shRNA in HepG2 cells

Data represent means ± SEM, standardized to U6Control-treated samples, of three independent experiments performed in duplicate. Samples were taken at the indicated times after transfection with HBV.

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<tr>
<th>Sample</th>
<th>Relative HBsAg in culture medium (%)</th>
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<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>U6S*</td>
<td>1.13 ± 0.92</td>
</tr>
<tr>
<td>U6Control</td>
<td>100 ± 6.43</td>
</tr>
<tr>
<td>Mock</td>
<td>112.35 ± 15.43</td>
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*Statistical differences between U6S and U6Control were tested using Student’s t test, P≤0.005.

Fig. 3. Analysis of HBV mRNA levels in HepG2 cells pretreated with shRNA-expressing baculovirus. (a) Total RNA was harvested from shRNA-pretreated HepG2 cells at the indicated times after initiation of HBV replication by an HBV-expressing baculovirus and analysed by Northern blotting. The positions of the major HBV transcripts and GAPDH are indicated. Time p.t., Time post-transduction (days). (b) Results were normalized to GAPDH, standardized to U6Control-treated cells and are represented as means ± SEM of three independent experiments (n=6). Data that are considered statistically significant are indicated by asterisks: *, P≤0.05; **, P≤0.005.
The effect of U6S on HBsAg levels secreted into the culture media was determined. Results were calculated using data from three independent experiments and are presented as a normalized mean relative to U6Control-treated cells. U6S-expressing baculovirus significantly inhibited HBsAg levels over time compared with U6Control-treated cells (Table 2).

**Fig. 4.** Southern blot analysis of subcultured HBV-infected HepG2 cells and analysis of baculovirus DNA. HBV RI (a), EC (b) and CCC (c) DNA was extracted, at the time points indicated, from HepG2 cells that were subcultured 1:4 10 days after initiation of HBV replication with an HBV-expressing baculovirus. RC, DS and SS bands are indicated. (d) Quantification of the various HBV DNA species. Levels of HBV DNA in subcultured cells were standardized to day 10 unsplit cells. (e, f) Input baculovirus DNA was extracted from subcultured and unsplit HepG2 cells at the times indicated and analysed by Southern blot. The Southern blot (e) and quantification of baculovirus input DNA (f) are shown. Times post-transduction (p.t.) are indicated and times post-subculture (p.s.) are indicated by a preceding 'S' and/or in parentheses (days).
DISCUSSION

Two different HBV/HepG2 cell model systems were used to evaluate the effects of a specific shRNA on HBV replication. To examine the effects on establishment of HBV replication, the original HBV recombinant baculovirus/HepG2 system that we previously reported and characterized was used (Abdelhamed et al., 2002, 2003; Delaney & Isom, 1998; Heipertz et al., 2007). The HBV baculovirus system makes use of the fact that HepG2 cells can support high levels of HBV replication and the baculovirus can efficiently transduce HepG2 cells. The construct contains a 1.3 unit length

**Fig. 5.** Analysis of HBV DNA levels in subcultured HBV-infected HepG2 cells treated with shRNA-expressing baculovirus. HBV RI (a, b), EC (c, d) and CCC (e, f) DNA was extracted from HepG2 cells transduced with the specified shRNA-expressing baculovirus at the indicated times after initiation of HBV replication with an HBV-expressing baculovirus. In (a), (c) and (e), results are standardized to U6Control-treated cells and represented as means ± SEM of three independent experiments; n = 6 unless otherwise indicated (in parentheses on the bars). Data that are considered statistically significant are indicated by asterisks: *, P < 0.05; **, P < 0.005. (b), (d) and (f) show representative Southern blots. Bands indicate RC, DS, SS and CCC HBV DNA. Times post-transduction (p.t.) are indicated and times post-subculture (p.s.) are indicated by a preceding ‘S’ and are in parentheses (days).
HBV genome, strain ayw, and only HBV regulatory elements. HBV genetic information is delivered by the baculovirus to the HepG2 cell nucleus. The process of receptor-mediated HBV infection, uncoating and transport of the partially double-stranded DNA to the nucleus are bypassed, and as such, the system does not recapitulate the entry process of natural HBV replication. Within 10 h of transduction, HBV transcripts and proteins are made and HBV capsids are found in the cytoplasm (R. A. Heipertz, Jr. and H. C. Isom, unpublished data). Intracellular RI are detected during the first day after transduction, and EC and nuclear HBV CCC DNA are not detected before day 2 after transduction. When HepG2 cells are transduced with a recombinant baculovirus containing a reporter gene under the control of a mammalian promoter, such as CMV promoter-driven lacZ, the expression of the gene product is transient, peaking at 48–72 h post-transduction and becoming undetectable by 5–6 days post-transduction. In contrast, when HepG2 cells are transduced with HBV baculovirus, HBV replication, including production of CCC DNA, is easily detectable for at least 30–35 days post-transduction (Abdelhamed et al., 2002).

**Table 2.** Inhibition of HBsAg secretion by shRNA in subcultured HepG2 cells

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<tr>
<th>Sample</th>
<th>Relative HBsAg in culture medium (%)</th>
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<tr>
<td></td>
<td>Day 2</td>
</tr>
<tr>
<td>U6S*</td>
<td>42.78 ± 9.02</td>
</tr>
<tr>
<td>U6Control</td>
<td>100 ± 4.53</td>
</tr>
<tr>
<td>Mock</td>
<td>85.92 ± 13.15</td>
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*Statistical differences between U6S and U6Control were tested using Student’s t test, *P* ≤ 0.005.
a switch occurs, with HBV transcription being driven from CCC DNA by 5–10 days post-transduction. In the HBV baculovirus system, HBV capsids deliver newly synthesized HBV genomes back into the nucleus.

The present study examined the effects of an anti-HBsAg shRNA-expressing baculovirus on HBV replication. Four regions of the HBV genome were chosen as potential RNAi target areas. Treatment of cells with U6S, a sequence that targets the HBsAg, core, HBeAg and polymerase mRNAs, as well as the HBV pgRNA, resulted in significant suppression of viral CCC DNA when administered to cells 24 h prior to transduction with HBV baculovirus, although not to the same magnitude as was observed for RI and EC DNA. These data demonstrated that U6S is capable of diminishing establishment of HBV replication in vitro. Although U6S in this study was developed independently, the powerful antiviral effects of this sequence have been previously reported and are comparable to those described in this study. For example, a greater than 98 % inhibition of HBsAg and 80 % inhibition of HBV RNA were achieved when HepG2.2.15 cells were transduced with an adeno-associated virus shRNA expression vector (Moore et al., 2005). The present study is unique because it is the first to evaluate the effects of this shRNA on HBV CCC DNA. The finding that pretreatment with U6S had a greater effect on RI or EC than CCC HBV DNA was similar to the findings regarding the effects of lamivudine or L-FMAU on HBV replication using the HBV baculovirus system (Abdelhamed et al., 2002, 2003; Delaney et al., 1999).

Although the ideal goal is to develop an antiviral agent or combination of antiviral agents that will eliminate ongoing chronic HBV infection, there is value in identifying therapies that can block the initiation of HBV infection. Preventing recurrent HBV infection in liver transplant patients is a vital component of successful therapy, but few effective treatment options are currently available (O’Grady et al., 1992; Todo et al., 1991). Sustained administration of HBV immunoglobulin and nucleoside analogues prior to and following liver transplantation has been shown to increase survival rates (Han et al., 2000; Markowitz et al., 1998; Zheng et al., 2006), but these agents can select for drug-resistant mutants (Melegari et al., 1998). The data reported in the current study indicate that pretreatment with anti-HBV shRNA U6S can delay or reduce establishment of replication in vitro. Unlike nucleoside analogues, which inhibit HBV only at the level of reverse transcription, the U6S sequence not only diminishes HBV DNA levels but also inhibits the production of HBV RNA and protein (Cheng et al., 2005; Lau et al., 2000; Wu et al., 2005).

Many studies on HBV replication in vitro rely on cell lines containing integrated copies of the viral genome or cells transduced with HBV expression vectors. Although these systems are useful for studying the HBV life cycle and therapeutic intervention, they are incapable of modelling true chronic HBV infection. Because naturally occurring HBV transcription and replication are driven by nuclear HBV CCC DNA, it is crucial to assess the efficacy of anti-HBV agents in a system that mimics these conditions. We have modified the original HBV baculovirus system to include the use of subculture at day 10 post-transduction to generate an HBV system in which transcription is driven from CCC DNA, and HBV replication is ongoing. This is quite different from what is observed when HBV replication is initiated by transduction with HBV baculovirus, where EC and CCC DNA are not detectable before day 2 post-transduction (Abdelhamed et al., 2002; Delaney & Isom, 1998). Using this model, we have demonstrated that although the U6S shRNA sequence significantly reduces HBV transcripts and inhibits HBV RI and EC DNA in chronically infected cells, it does not affect CCC DNA levels. Indeed, these findings could be considered negative since the shRNA had no effect on preformed CCC DNA levels, but these data are highly important because they re-emphasize the fact that HBV CCC DNA is resilient to antiviral attack, and support the concept that once formed, CCC DNA is extremely stable. In addition, U6S does not completely inhibit production of intercellular RI and virions and RI DNA-driven recycling; therefore, highly stable CCC DNA pools are replenished, thus maintaining a constant level of nuclear CCC DNA. It has been reported previously that transfection of HepG2.2.15 cells with vector-based siRNAs targeted to the HBV core nuclear localization signal, either alone (Li et al., 2007) or in combination (Xin et al., 2008), inhibit HBV CCC DNA, as measured by RT-PCR. It is not possible to compare these studies with our data due to differences in siRNA target sequences, in vitro HBV model systems, quantification and methods of CCC DNA extraction, specifically, isolation of CCC DNA from the culture medium (Li et al., 2007) versus extraction of CCC DNA from the nuclear fraction of infected cells (this study).

Success in truly combating chronic HBV will require targeting of multiple different stages in the HBV life cycle. The unique method of action and demonstrated efficacy of RNAi-based therapies make them an attractive complement to existing therapeutic regimes. Recombinant baculovirus vectors are valuable tools for research because of their ability to efficiently and reproducibly transduce cells in culture and to modulate gene expression. They also have therapeutic potential. Baculovirus expressing the diphtheria toxin under control of the glial fibrillary acidic protein promoter has been used successfully to inhibit the growth of glioma xenografts in rat brain (Wang et al., 2006). Several groups have also been able to elicit both humoral and cellular immune responses in vivo using recombinant baculovirus expression and display vectors (Abe et al., 2003; Kim et al., 2007; Strauss et al., 2007).

The goals of this study were to evaluate the use of RNAi technology on blocking the initiation of HBV replication and on inhibition of chronic ongoing HBV replication. In the process of addressing the latter question, the HBV baculovirus subculture system was established and its efficacy as a model for antiviral testing using an shRNA was evaluated. The HBV baculovirus system has specific
advantages for drug studies because it is possible to measure the effect of antiviral agents on all aspects of the HBV life cycle, including CCC DNA, and to do so quantitatively. HBV CCC DNA is present at sufficient levels to be detected by Southern blot analysis in both the original and the subculture system. In addition, as demonstrated in this report, agents delivered by gene therapy can also be evaluated; specifically, the reseeding process after subculture makes it possible to reproducibly carry out a supertransduction with a second recombinant baculovirus expressing a DNA sequence of interest, in this case an shRNA. The HBV baculovirus subculture system has the potential to be used in the future to analyse the efficacy of nucleoside analogues, novel small molecule inhibitors, RNAi or other agents, alone or in combination, on chronic HBV replication.

ACKNOWLEDGEMENTS


REFERENCES


