Inhibition of woodchuck hepatitis virus gene expression in primary hepatocytes by siRNA enhances the cellular gene expression

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Abstract

Small interfering RNA (siRNA) has been shown to be active to inhibit the hepatitis B virus gene expression and replication in transient and stable transfection systems. Here in primary hepatocytes prepared from naturally woodchuck hepatitis virus (WHV)-infected woodchucks, four siRNAs targeting the WHV preS1, S, C, and X region led to a depletion of WHV transcripts and replicative intermediates with different kinetics and a decreased production of viral particles. Two siRNAs targeting WHV S and X region had the highest efficacy to deplete 70% of WHV transcripts and replicative intermediates. In addition, siRNA-mediated suppression of WHV enhanced the expression of cellular genes like MxA and MHC I. Specific siRNAs are able to inhibit the hepadnaviral replication and enhance the expression of cellular genes relevant for antiviral actions. Thus, siRNAs might be useful as novel antiviral agents for the treatment of chronic HBV infection.

Introduction

About 350 million people are chronically infected with hepatitis B virus (HBV) world-wide. These patients have a relatively high risk of developing end-stage liver diseases, such as liver cirrhosis and hepatocellular carcinoma (Seeger and Mason, 2000). Despite significant progress in recent years, the currently available treatment regimens for hepatitis B such as interferon-α (IFN-α) or nucleoside/nucleotide analogues are costly and have limited long-term efficacy. Only about one-third of patients treated with IFN-α show a sustained response. Nucleoside or nucleotide analogues are not able to eliminate the virus completely in the vast majority of patients and may select resistant viral variants during the prolonged treatment (Locarnini and Mason, 2006; Marcellin, 2002). Therefore, it is necessary to develop new antiviral strategies against HBV.

RNA interference (RNAi) may provide a promising approach for the specific treatment of HBV infection. It is mediated by small interfering RNAs (siRNAs) of a length of 21 to 23 nucleotides (Hannon, 2002) that lead to the sequence-specific degradation of homologous messenger RNAs (mRNAs). Using chemically synthesized or vector-expressed siRNAs, many clinically relevant viruses including the human immunodeficiency virus, HBV, and hepatitis C virus could be inhibited in vitro (Randall and Rice, 2004; Stevenson, 2003; Wu and Nandamuri, 2004). A number of recent studies have demonstrated the effectiveness of specific siRNAs for inhibiting the HBV gene expression and viral replication (Giladi et al., 2003; Guo et al., 2005; Hamasaki et al., 2003; Klein et al., 2003; Konishi et al., 2003; McCaffrey et al., 2003; Morrissey et al., 2005; Shlomai and Shaul, 2003; Uprichard et al., 2005; Wu et al., 2005). Yet, RNAi has not been tested in a natural infection system with a complete viral life cycle so far.

The woodchuck hepatitis virus (WHV) is a member of the family Hepadnaviridae discovered in 1978 (Summers et al., 1978). WHV causes acute and chronic infections in woodchucks (Marmota monax) like HBV in humans. The woodchuck model has been proven to be an informative model for studies on hepadnaviral infection and pathogenesis and for evaluation of antiviral drugs (Lu et al., 2007; Roggendorf and Lu, 2005). In this study, we examined the inhibition of the WHV gene expression and replication in WHV-infected primary woodchuck hepatocytes (PWHs) by specific siRNAs. In addition, we investigated whether the suppression of viral gene expression modulates the expression of host genes with antiviral and immunological functions.

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Fig. 1. Inhibition of the WHV gene expression by specific siRNAs. (a) A schematic presentation of the open reading frames on the WHV genome and the sites targeted by siRNAs used in this study. (b) The ability of the selected siRNAs to knock down the specific WHV transcripts. HepG2 cells were co-transfected with 20 nM of siRNA and 1 μg of the expression plasmids for selected targets. The WHV gene transcripts were detected 72 h later by Northern blot. The quantification of the signals was performed by using Phosphor-Imager. (c) BHK cells were co-transfected with 20 nM of siRNA and 1 μg of the expression plasmids for selected targets. WHsAg and WHcAg were detected by immunofluorescence staining with specific antibodies. The expression of EGFP was directly examined by fluorescence microscopy. RDLU, relative digital light units.
Results

siRNAs targeting the WHV preS1, S, C, and X gene inhibit WHV gene expression

Four siRNAs targeting to the WHV genome were designed by using the HiPerformance siRNA Design Algorithm (Fig. 1a). The specific inhibitory effect of siRNAs was demonstrated by co-transfection of siRNAs with the respective expression plasmids for WHpreS1, WHsAg, WHcAg, and WHxAg-EGFP into HepG2 cells or BHK cells. In the HepG2 cells, expression of the specific WHV mRNAs was decreased by co-transfection with the respective siRNAs (Fig. 1b). Two siRNAs siWHs and siWHx were highly efficient to reduce the specific mRNAs to 24% and 27% of the mock control while siWHpreS1 and siWHc had only moderate effects (Fig. 1b).

Additionally, the inhibitory effects of WHV-specific siRNAs were investigated on the protein level by the direct detection of WHV proteins WHsAg and WHcAg by immunofluorescence staining in BHK cells. The expression of EGFP by the WHxAg-EGFP fusion construct was examined by fluorescence microscopy. Consistent with the detection of specific mRNAs, the WHV proteins expression was decreased by co-transfection with specific siRNAs (Fig. 1c).

Specific siRNAs lead to the depletion of WHV transcripts and replicative intermediates in PWHs

PWHs from chronically infected woodchucks are uniformly infected by WHV and contain usually a high steady-state level of WHV specific transcripts and replicative intermediates. It has been shown that there are two major transcripts, 2.1 and 3.7 kb in length found in the liver samples from chronic WHV–infected woodchucks (Moroy et al., 1985). The 2.1 kb RNAs correspond to the transcripts for major WHsAg while the 3.7-kb RNA is involved in the expression of other viral proteins. It is of interest to examine whether specific siRNAs are able to deplete the WHV RNAs and replicative intermediates. PWHs with WHV infection were transfected with specific siRNAs or control siRNA, respectively. The levels of WHV transcripts and WHV DNA were assayed on days 2, 4, and 7 after transfection by Northern blot and Southern blot, respectively. The reduction of WHV transcripts by specific siRNAs was readily detectable on day 2 (Fig. 2a).

Fig. 2. Inhibition of the WHV gene expression and replication in PWHs by specific siRNAs. PWHs were transfected with 100 nM of siWHpreS1, siWHs, siWHc, siWHx, or a control siRNA siGFP. The WHV transcripts (a) and replicative intermediates (b) were analyzed on days 2, 4, and 7 post transfection by Northern blot and Southern blot, respectively. A representative result is shown. The data presented as means ± standard deviation from three independent Southern blotting analysis were also shown (b lower panel). S1, siWHpreS1; S, siWHs; C, siWHc; X, siWHx; N, siGFP as a negative control; RDLU, relative digital light units.
Two siRNAs, siWHpreS1 and siWHs, were directed to the transcripts of WHV surface proteins. The treatment with siWHpreS1 led to the reduction of WHV 3.7 kb transcripts while the amount of the 2.1 kb transcripts remained unchanged, since siWHpreS1 has no target site on the 2.1 kb transcripts. In addition, both 3.7 and 2.1 kb transcripts were decreased after the transfection with siWHs and siWHx as well. The siRNA siWHc was less effective to knock down the WHV transcripts. The inhibitory effect on WHV-specific mRNAs reached a maximum on day 4. The transfection with siWHs and siWHx led to a reduction of WHV mRNA to 35% and 31%, respectively. The effect of siRNAs lasted at least until day 7 (also see below).

After transfection with WHV-specific siRNAs, WHV replicative intermediates in PWHs decreased with a delay compared with the kinetics of reduction of WHV mRNAs. This can be explained by the fact that siRNAs do not act directly on the level of the WHV DNA intermediates. The decrease of WHV replicative intermediates was mainly caused by the reduced formation of new WHV ss DNA from pregenomic RNAs. Consistently, transfection with two potent siRNAs siWHs and siWHx reduced the amounts of WHV replicative intermediates in PWHs to a level of 37% on day 7 as compared with the mock control (Fig. 2b). The other two siRNAs were less effective.

Furthermore, the release of viral particles into the culture supernatants was assessed. WHV DNA in culture supernatants was extracted and quantified by real-time PCR. A single transfection of siWHpreS1, siWHs, and siWHx reduced the WHV DNA levels in the culture supernatants. A reduction of more than 50% of the WHV DNA level was measured on day 2 after treatment with siWHs and siWHx and reached a level of about 90% suppression on the day 4 (Fig. 3). A repeated transfection of siRNAs 24 h after the first transfection could further enhance the silencing effect and led to a 90% reduction of the WHV DNA concentration in the cell culture supernatants on day 7 (data not shown).

Single transfection of siRNA has only a transient inhibitory effect on WHV replication

As siRNAs have only limited stability in cells, the RNAi effect ceases over time and new transcripts can be produced from WHV cccDNAs (Bartlett and Davis, 2006). Thus, we examined whether new WHV replicative intermediates are formed in PWHs treated with specific siRNAs. PWHs were transfected with siRNAs and the amounts of WHV replicative intermediates in PWHs were determined up to day 14 after transfection. As already shown previously, a single transfection with siWHx led to a reduction of WHV replicative intermediates up to day 14 (Fig. 4). However, the level of WHV replicative intermediates in siWHx-treated PWHs returned gradually to a high level on day 14, suggesting that the therapeutic use of siRNAs against HBV will face similar problems with the persistence of cccDNAs as observed during the treatment with nucleoside analogues.

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**Fig. 3.** Inhibition of the production of WHV viral particles released into the cell culture supernatants by specific siRNAs. PWHs were transfected with 100 nM of siWHpreS1, siWHs, siWHc, or a control siRNA siGFP. The copy numbers of WHV progeny DNA secreted into culture supernatants were determined on days 2, 4, and 7 post transfection by real-time PCR. * Compared with mock control, *p* < 0.05; ** compared with mock control, *p* < 0.01.

**Fig. 4.** Suppression and rebound of the WHV replication after a single siRNA transfection. PWHs were transfected with 100 nM of siWHx or siWHc. WHV replicative intermediates were examined on days 2, 4, 7, 10, and 14 by Southern blot analysis (a). The signals detected in Southern blot were analyzed, and the relative strength of signals was calculated by setting the mock transfection control as 100% (b). X, siWHx; C, siWHc.
Silencing of WHV gene expression and replication enhances the expression of cellular antiviral proteins

It has been shown that hepadnaviruses may influence the expression of IFN-stimulated genes (ISGs) in hepatocytes (Foster et al., 1991; Twu and Schloemer, 1989; Wu et al., 2007). Thus, suppression of the WHV gene expression and replication may lead to changes in the expression of these genes. Therefore, we focused on MxA and MHC I, ISGs known to be involved in antiviral activities and specific immune responses. The expression of MxA and MHC I in siRNAs-transfected PWHs was determined by one-step real-time RT-PCR. siRNAs had no effect on the MxA and MHC I expression in primary hepatocytes from naïve woodchucks (Fig. 5a). By contrast, a significant increase of the MxA and MHC I expression occurred in WHV-infected hepatocytes after transfection with siWHs and siWHx that effectively reduced the WHV gene expression (Fig. 5a). siWHc and the control siRNA (siGFP), however, had no or little effect on MxA and MHC I expression in WHV-infected primary hepatocytes. The enhanced expression of MxA and MHC I occurred on the day 2 after transfection with siWHs and siWHx. However, the MxA expression in siRNA treated hepatocytes decreased gradually and returned to the base line levels within 10 days (Fig. 5b). Interestingly, a repeated transfection with siWHx stimulated MxA expression again. The mRNA expression levels of some other cellular genes were also investigated, accordingly IFN-β, OAS, IP-10, and TNF-α mRNA were all significantly upregulated in siWHs and siWHx-treated WHV-infected PWHs (data not shown). These results demonstrated that the silencing of the WHV gene expression enhances the cellular functions that contribute to the immunological control of viral infections.

We further examined whether the IFN signaling pathways are modulated by silencing of WHV replication. Two days post transfection with siWHx, the STAT-1 expression was elevated in PWHs (data not shown). However, transfection of siRNAs did not affect the

![Fig. 5. Enhancement of the cellular gene expression in PWHs by silencing of WHV. (a) PWHs were transfected with 100 nM of siWHpreS1, siWHs, siWHc, siWHx, or a control siRNA siGFP. The expression levels of MxA and MHC I in siRNAs-transfected PWHs were determined by one-step real-time RT-PCR on day 2 post transfection. (b) Kinetics of the MxA expression in PWHs upon siRNA transfection. PWHs were left untreated (○), transfected with siWHx (□), or control siRNA siGFP (△). A repeat transfection of siWHx (■) and siGFP (▴) was performed on day 7 after the first transfection. The expression levels of MxA were determined by one-step real-time RT-PCR on days 2, 4, 7, and 10 post transfection. WHV (+) PWH, PWH with WHV infection; WHV (−) PWH, PWH without WHV infection; * Compared with mock control, p < 0.05; arrows indicate the time points of transfection.](image1)

![Fig. 6. Synergistic effect of siRNAs and ETV on WHV replication. (a) PWHs were treated with 100 nM of siRNAs, ETV, or a combination of siRNA and ETV for 2 days. (b) PWHs were pretreated with 100 nM of ETV for 10 days, then transfected with 100 nM of siRNAs and incubated in normal maintaining medium for further 7 days. The WHV replicative intermediates and transcripts were analyzed by Southern blot and Northern blot analysis, respectively. A representative experiment is shown. S1, siWHpreS1; S, siWHs; C, siWHc; X, siWHx; N, siGFP as a negative control; RDLU, relative digital light units.](image2)
expression of TRIF, MyD88, and IRAK1 (data not shown). Furthermore, Bay11-7082, an NF-kappa B inhibitor did not block the stimulation of MxA in PWHs (data not shown).

**A synergistic action of siRNAs and ETV on the inhibition of WHV replication**

Nucleoside analogues like ETV are effective antivirals to inhibit the activity of hepadnaviral polymerases and prevent the formation of viral replicative intermediates (Hadziyannis, 2006). Treatment of WHV-infected PWHs with ETV at a concentration of 100 nM significantly inhibited WHV replication within 2 days, while combination of siWHx and ETV exhibited more potent inhibition on WHV replication than ETV or siRNA treatment alone (Fig. 6a). Treatment of PWHs with ETV for 10 days led to a strong reduction of WHV replicative intermediates to a level below 20% of untreated controls (Fig. 6b upper panel). The levels of WHV transcripts remained unchanged by ETV treatment, while the transfection with siRNAs resulted in a reduction of WHV RNAs (Fig. 6b lower panel). The inhibitory effect of siRNAs on the WHV replication could be seen after the discontinuation of ETV treatment. The WHV replication rebounded significantly 7 days after ETV discontinuation but remained at the low levels if the hepatocytes were treated with siWHs and siWHx (Fig. 6b upper panel).

Furthermore, we tested whether inhibition of WHV replication by ETV may have an influence on the expression of MxA as observed for the use of WHV-specific siRNAs. ETV treatment did not lead to induction of MxA in PWHs (Fig. 7), while their expression was enhanced after transfection with siWHx but not siWHc. These results indicated that siRNAs could contribute to the antiviral therapies through different ways and act synergistically with ETV.

**Discussion**

In this study, we demonstrated that WHV-specific siRNAs were able to reduce the levels of WHV mRNAs in primary hepatocytes naturally infected with WHV. Four siRNAs targeting the different regions of the WHV genome were used in this study. The siRNAs targeting the WHV S and X region had the highest efficacy while siRNA against the WHV core region was not effective, probably due to the inappropriate target sequence. The siRNA to the WHV preS region was able to induce the degradation of the specific 3.7 kb RNAs, while it was less potent in suppressing WHV replicative intermediates, compared to siWHx and siWHc. In addition, our results also indicated that siRNAs and nucleoside analogues may act synergistically in suppressing WHV replication. The control siRNA had slight unspecific effects on WHV replication. However, this effect was not significant when compared with mock control.

There are some interesting features in the action of effective siRNAs on WHV-infected PWHs. First, the amounts of WHV RNAs, replicative intermediates, and WHV DNAs in culture supernatants changed with different kinetics. The decrease of the levels of WHV-specific RNAs was readily measurable on day 2. However, the depletion of WHV replicative intermediates became visible on day 4, thus with a delayed kinetics compared to that of WHV transcripts. Obviously, a pool of WHV replicative intermediates was present in persistently infected PWHs and needed about more than 2 days to be depleted. The production of viral particles was decreased on day 2 as the amounts of WHV RNAs were reduced in PWHs, probably due to the reduced expression of WHV proteins. Second, the amounts of viral replicative intermediates returned to basal levels within two weeks. Two factors determine the kinetics of viral rebound. One factor is the half-life of siRNAs in PWHs. However, it is difficult to determine the half-life of siRNA in transfected cells and to correlate with the RNAi effects (Chiu and Rana, 2003). The formation of new pregenomic RNAs from the WHV cccDNA pool in PWHs will determine the final steady-state level of WHV replicative intermediates. Thus, repeated applications of siRNAs or use of modified siRNAs with a longer half-life would be necessary for clinical use.

Furthermore, the knock down of WHV gene transcripts by specific siRNAs led to an enhanced expression of cellular genes like MxA and MHC I, which are related to specific immune responses and antiviral functions. An increased expression of MxA and MHC I was only detected in WHV-infected PWHs but not in naive PWHs. Thus, we could exclude that the biochemical properties of siRNAs itself were responsible for IFN induction. In addition, upregulation of the MxA and MHC I expression occurred only if the siRNAs were effective to degrade WHV RNAs. For example, siWHc did not induce an increase of the MxA and MHC I expression in WHV-infected PWHs, as this siRNA was inactive. Therefore, the mechanisms leading to the enhanced cellular gene expression by WHV-specific siRNAs need to be examined further. One hypothesis is that the knock down of WHV transcripts would reduce the amount of WHV proteins and therefore facilitate the cellular gene expression. Early reports indicated that HBV polymerase may inhibit the IFN signaling pathway (Wu et al., 2007). Further, HBV may counteract the host antiviral effector mechanisms by down-regulating the IFN-inducible MxA promoter through direct interaction of precore/core proteins or by inhibiting proteasome activities in an HBx-dependent manner (Fernandez et al., 2003; Zhang et al., 2004). In this context, knock down of the viral gene expression would relieve these inhibitory effects on the cellular gene expression. Another hypothesis to be investigated is that the induction of the cellular genes may occur due to the triggering of IFN responses by degradation products of WHV RNAs during RNAi. It has been reported that a cleavage of cellular RNAs by RNase L produces small RNAs that are able to activate IFN-β (Malathi et al., 2007). It should be investigated if a siRNA-directed RNA degradation would also produce small RNA fragments and trigger the activation of IFN-β gene. This process could not yet be examined in woodchuck cells due to lack of woodchuck specific reagents. It is noteworthy, however, that an enhanced expression of MxA and MHC I by siRNA treatment does not occur in established human hepatoma cell lines with HBV replication (Meng and Lu, unpublished results). This may be due to the defective IFN signaling pathways in such hepatoma cells (Stojdl et al., 2000). However, early publications indicated that some other cell lines may be suitable for such studies (Bridge et al., 2003; Sledz et al., 2003).

Though the mechanisms leading to the enhanced cellular gene expression need further studies, this effect itself may facilitate the immunotherapy and lead to an enhanced antiviral activity in vivo. In chronically HBV-infected patients, there is a deficiency of specific cellular responses to HBV proteins (Chisari and Ferrari, 1995; Rehermann, 2007). The early attempts to stimulate the specific cellular responses in such patients by conventional HBV vaccines or DNA vaccines had only limited effects (Mancini-Bourigne et al., 2004).
It is proposed that a pretreatment of chronically HBV-infected patients with antiviral drugs would enhance the effect of therapeutic vaccines, as an effective antiviral treatment with lamivudine would restore the cellular immune responses in patients to a limited extent (Bonì et al., 1998, 2001, 2003). A combined treatment with antiviral drugs and therapeutic vaccines has not yet been successfully tested in clinical trials (Dahmen et al., 2002; Horiike et al., 2005; Vandepadepere et al., 2007). In the woodchuck model, such combined approaches have shown an improved antiviral effect (Lu et al., 2008; Menne et al., 2007). An enhancement of cellular IFN responses by siRNA may be beneficial and is worth future investigation.

Taken together, we demonstrated the potential usefulness and limitation of siRNAs for inhibition of hepadnaviral gene expression and replication in naturally WHV infected hepatocytes. The ability of siRNAs to modulate the cellular gene expression specifically by suppression of hepadnaviral gene expression and replication represents a unique aspect and provides new options for treatment of chronic HBV infection.

Materials and methods

siRNAs and entecavir (ETV)

Four siRNAs were designed by using the HiPerformance siRNA Design Algorithm (Qiagen, Düsseldorf, Germany) in combination with the algorithm described by Reynolds et al. (Reynolds et al., 2004). The sequences of the siRNAs are given according to the WHV59 genome sequence (GenBank accession no. M19183). The selected siRNAs, designated as siWHpreS1, siWHs, siWHc, and siWHx, have the specific target sequences within the WHV preS1, S, C, and X gene, respectively (Fig. 1 and Table 1). siGFP, a siRNA against green fluorescent protein, was used as the control (Table 1). All siRNAs were purchased from Qiagen. ETV was purchased from Bristol-Myers Squibb (Munich, Germany).

Woodchucks

All animals were purchased from North Eastern Wildlife (Ithaca, NY, USA) and kept in the center of animal laboratory of the University of Essen. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were reviewed and approved by the local Animal Care and Use Committee of the district government (Düsseldorf, Germany) for the Care and Use of Laboratory Animals and were reviewed and approved by the local Animal Care and Use Committee of the district government (Düsseldorf, Germany).

Table 1 siRNAs target sequences

<table>
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<th>Designation</th>
<th>Target sequences</th>
<th>Locationa</th>
<th>WHV gene</th>
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<tr>
<td>siWHpreS1</td>
<td>5′-AGCACTACAGCTTGGGATAC-3′</td>
<td>3279-3299</td>
<td>PreS1</td>
</tr>
<tr>
<td>siWHs</td>
<td>5′-ACGAGACTGTCGCGAGAAG-3′</td>
<td>3292-3311</td>
<td>S</td>
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<tr>
<td>siWHc</td>
<td>5′-AAAGAGCATAATGATAGTAC-3′</td>
<td>2263-2283</td>
<td>C</td>
</tr>
<tr>
<td>siWHx</td>
<td>5′-AAAGATCATAATTTATTAACAA-3′</td>
<td>1827-1847</td>
<td>X</td>
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* The positions of the target sequences according to the WHV sequence M19183.

Primary hepatocytes culture and siRNA transfection

Primary hepatocytes from WHV-infected and uninfected woodchucks were prepared by liver perfusion with collagenase IV (Sigma, Munich, Germany) as described previously (Baccarani et al., 2003). The viability of prepared hepatocytes was assessed by trypan blue dye exclusion and exceeded 80% in all experiments. The cells were plated at a density of 1.5×10⁶ cells per well on collagen I-coated six-well plates in Williams medium (Gibco, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Gibco), 1 mg/ml of hydrocortisone (Sigma), 10 μg/ml of insulin (Serva, Amsterdam, The Netherlands), 50 μg/ml of gentamycin (Sigma), 50 IU/ml of penicillin-streptomycin (Gibco), 2 mM glutamine (Gibco), 1.25 mg/ml of inosine (Serva), and 1% DMSO (Merck, Darmstadt, Germany).

Transfection of primary hepatocytes with siRNAs was carried out on day 2 of in vitro culture except specification. Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) was used according to the manufacturer’s instructions. 150 pmol siRNA and 5 μl of Lipofectamine 2000 per well were applied in a final volume of 1.5 ml Opti-MEM. After 5 h, the medium was replaced by fresh culture medium.

To ensure highly efficient siRNA delivery into hard-to-transfect PWHs, a high amount of siRNA is needed (Gilot et al., 2005). A dose-finding experiment suggested that siWHx inhibited the WHV gene expression and replication in a dose-dependent manner. 100 nM of siRNAs were used in the present experiments. Although higher concentration than 100 nM led to more potent effect on the mRNA level, no more reduction on WHV replication was observed (data not shown).

Construction of expression vectors for WHV preS1, surface antigen (WHsAg), core protein (WHcAg), and X protein (WHxAg)-enhanced green fluorescent protein (EGFP) fusion

The plasmids expressing WHsAg and WHcAg were described previously (Lu et al., 1999). To construct the vector expressing the WHV preS1 protein, the WHV genome region (nt 2992–964) was amplified by PCR with the primers whpres1 and whs2 (Table 2) and cloned into the pCR2.1 vector (Invitrogen). The fragment containing the large WHsAg gene was isolated by digestion with EcoRI and inserted into the EcoRI site of the pcDNA3 vector (Invitrogen). The generated plasmid contained the large WHsAg gene under the control of the cytomegalovirus promoter. The region comprising the WHV X region was amplified with the primers whx-1 and whx-2 (Table 2), and cloned into the pCR2.1 vector. The clone sequence was verified by DNA sequencing. The fragment containing the WHX region was cut by EcoRI and cloned into the EcoRI site of pIRE52-EGFP vector (Clontech, Heidelberg, Germany).

Table 2 Primers used for amplification of WHV preS1, X, and C gene and real-time RT-PCR assay

<table>
<thead>
<tr>
<th>Amplicon</th>
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<th>Position</th>
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<td>WHV C gene</td>
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<td>wβ-actin-A1</td>
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<tr>
<td>Woodchuck MHC I</td>
<td>wMHC-I-s1</td>
<td>Sense</td>
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<td>AF146091</td>
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<tr>
<td>Woodchuck MxA</td>
<td>wMxA-s1</td>
<td>Sense</td>
<td>5′-CAGAAGCAGAGCACAGA-3′</td>
<td>490</td>
<td>AF146091</td>
</tr>
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</table>

* The underlined part of primer wc-149s indicates the specific cleavage sites of restriction enzyme BgIII.
Cell cultures, transfection, and immunofluorescence staining

Culturing and transfection of BHK and HepG2 cells were carried out as described previously (Zheng et al., 2002). To detect WHSAg and WHAg, immunofluorescence staining was performed with monoclonal mouse anti-WHPreS (Waters et al., 2001; Zheng et al., 2002) or anti-WHAg antibodies (Zhang et al., 2006). The anti-WHPreS antibody recognizes the middle WHSAg that contains the major WHSAg as a domain. The expression of EGFP by the WHX-EGFP fusion was determined by direct observation and photography under a fluorescence microscope with an excitation wavelength of 490 nm.

Isolation and analysis of viral RNA and WHV replicative intermediates

Total RNA was extracted from hepatocytes with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Northern blot analysis was carried out by agarose-glyoxal method according to published protocols (Meng et al., 2008). WHV replicative intermediates were purified from intracellular core particles according to the protocol described for HBV previously (Meng et al., 2008). The isolated WHV DNA was subjected to agarose gel electrophoresis, followed by denaturation and Southern blotting. WHV RNA and DNA on Northern and Southern blots were detected by hybridization with a 32P-labeled full length HBV probe. The quantitative analysis of the signals on the Southern blots and Northern blots was performed by using a PhosphorImager (Cyclon, Parkard, Meriden, CT, USA). The total WHV replicative intermediates showed as a smear in the Southern blots including the three major forms: RC (relaxed circular), DL (duplex linear), and SS (single-stranded) DNA were quantified. The relative strength of signals (relative digital light units, RDLU) was calculated by setting the mock transfection control as 100%. The WHV specific gene transcripts in cotransfection experiments and the 3.7 and 2.1 kb RNAs in PWHs were quantified and normalized against β-actin. The RDLU was calculated by setting the mock transfection control as 100%.

Quantitation of WHV progeny DNA in culture supernatants

WHV progeny DNA in the culture supernatants was extracted using Qiamp DNA blood mini kit (Qiagen) and quantified as genome equivalents (GE) per ml by real-time PCR with LightCycler DNA master SYBR green kit (Roche Diagnostics, Mannheim, Germany). PCR was performed with the primers w1c and wc-149s (Table 2) (Lu et al., 2001; Zheng et al., 2002) on a LightCycler™ instrument (Roche) with 95 °C for 0 s, 53 °C for 10 s and 72 °C for 12 s. A plasmid containing a full length WHV genome was diluted and served as standard. The detection limit of this assay was 10^3 WHV GE/ml.

Real-time reverse-transcriptase (RT) PCR

Total RNA was purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. One-step real-time RT-PCR was performed with 100 ng of total RNA using Quantitect SYBR Green RT-PCR Kit (Qiagen) on a LightCycler™ instrument (Roche) with 50 °C for 10 min for reverse transcription followed by 45 cycles of PCR: 95 °C 10 s, 60 °C 30 s. The primers used are listed in Table 2. The relative mRNA copies were determined with a standard curve. The copy numbers of WHV genome was diluted and served as standard. The detection limit of this assay was 10^3 WHV GE/ml.

References


