Presence of Replicating Virus in Recombinant Hepadnavirus Stocks Results from Recombination and Can Be Eliminated by the Use of a Packaging Cell Line

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Mutant hepatitis B viruses are useful tools to study the viral life cycle and viral pathogenesis. Furthermore, recombinant hepatitis B viruses are candidate vectors for liver-directed gene therapy. Because wild-type viruses present in recombinant or mutant virus stocks may falsify experimental results and are detrimental for a viral vector, we investigated whether and to what extent wild-type virus is present in recombinant virus stocks and where it originates from. We took advantage of the duck model of hepatitis B virus infection which allows very sensitive detection of replication-competent viruses by infection of primary duck hepatocytes or of ducklings *in vivo*. Recombinant hepatitis B virus stocks contained significant amounts of wild-type viruses, which were most probably generated by homologous recombination between plasmids containing homologous viral sequences. In addition, replication-competent viral genomes were reconstituted from plasmids which contained replication-deficient but redundant viral sequences. Using a stable cell line for packaging of deficient viral genomes, no wild-type virus was detected, neither by infection of primary hepatocytes nor *in vivo*.

Hepadnaviruses are small, enveloped hepatotropic DNA viruses. The prototype member of this family is the human hepatitis B virus (HBV). Related hepadnaviruses were found in mammals (15, 17, 29) and birds (19, 25). The hepadnaviral genome consists of a partially double-stranded, relaxed circular (rc) DNA, which has a compact organization employing widely overlapping open reading frames (ORFs) and regulatory sequences. Replication of the viral genome occurs via reverse transcription of a pregenomic (pg) RNA intermediate (28), which, in addition to subgenomic RNAs, is transcribed from a free, covalently closed circular (ccc) form of the hepadnaviral genomic DNA (2).

Hepadnaviruses produced from cloned DNA basically serve two purposes: (i) when carrying a mutant viral genome, they are powerful tools for studying the viral life cycle; and (ii) when carrying a foreign gene, they are candidate viral vectors for liver-directed gene transfer. Recently, the ability of recombinant HBV and duck HBV (DHBV) to serve as vectors for a hepatocyte-specific gene transfer has been proven (21).

Hepadnaviruses are produced in cell culture by transfection of plasmids containing replication-competent viral DNA genomes. For replication to start from cloned hepadnaviral genomes, transcription of the more-than-genome-length viral pg RNA is required. Therefore, cloned DNA used to produce recombinant hepadnaviruses needs to contain terminally redundant genome sequences to serve as a transcription template. This was first obtained by using head-to-tail dimers of the HBV genome (18, 23, 25, 31). Subsequently, constructs were developed which more-efficiently initiated HBV replication. They contain a terminally redundant genome of HBV

from which the pg RNA is transcribed under control of a foreign promoter (12, 20). Furthermore, 1.2- to 1.5-fold genomes were developed which do not employ any foreign promoter to drive transcription of pg or subgenomic RNAs (9, 22, 30). Mutant hepadnaviruses are produced by introducing the respective mutant into one of these constructs. Due to the redundant sequences contained in all of these constructs, intramolecular or intermolecular recombination might occur.

To produce replication-deficient viral mutants or recombinant hepadnaviruses carrying a foreign gene, at least two plasmid constructs are required (3, 10, 21). The first plasmid expresses mutant or recombinant pg RNA containing all *cis*-acting sequences necessary for encapsidation and for reverse transcription into the DNA genome. This plasmid will be further referred to as the transfer plasmid. The second plasmid serves as a helper and must express and provide in *trans* all lacking viral proteins necessary for the formation of nucleocapsids, for reverse transcription, and for the envelopment of virions. In order to prevent encapsidation and reverse transcription of RNA expressed from the helper construct, the encapsidation signal ϵ (13) or direct repeats necessary for reverse transcription (10) are deleted. Because the plasmids used for the production of virus stocks either contain redundant viral sequences or share viral genome sequences, homologous recombination following transfection of the plasmids may result in accidental generation of wild-type or other replication-competent viruses (5).

The presence of wild-type virus in mutant virus stocks is a major problem because it may grow out with time and confound or falsify the results obtained. More importantly, if recombinant HBV is to be used as a gene transfer vector, the presence of wild-type HBV is a major safety issue. We therefore screened recombinant virus stocks for replicating wild-type virus. To be able to detect small amounts of replicating virus, we took advantage of the duck model of HBV infection

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because DHBV replicates and spreads efficiently in primary duck hepatocyte (PDH) cultures.

Here we show that recombinant virus stocks contain significant amounts of replicating virus generated by homologous recombination. We furthermore demonstrate that the amount of wild-type virus was significantly diminished when redundant DHBV sequences in the helper plasmids were deleted. Replicating virus was no longer detected when recombinant virus stocks were produced with a newly generated packaging cell line, neither by infection of hepatocyte cultures nor in vivo when ducklings were infected.

MATERIALS AND METHODS

Plasmid constructs. As a template for transcription of the recombinant pg DHBV RNA we used transfer plasmid pCD16-GFP as described recently (21). pCD16-GFP contains, under control of the cytomegalovirus immediate early promoter-enhancer, a terminally redundant genome of DHBV subtype 16 (DHBV16) (16) (Fig. 2). All nucleotide positions refer to the DHBV16 genome and are numbered from the unique *EcoRI* site. A DNA fragment containing the small envelope (S) gene from *KpnI* (nucleotide [nt] 1290) to *BstEII* (nt 1847) was replaced by a PCR fragment (733 nt) encoding a fluorescence-enhanced, red-shifted green fluorescent protein (GFP) (32) with expression expected to be driven by the S promoter (21). For construction of transfer plasmid pCD16-GFP-2, nt 1847 to 2020 were deleted and stop codons were introduced into the 5' ORFs of the core protein (nt 2660), the polymerase protein (nt 183), and the pre-S protein (nt 823 and nt 1165) (see Fig. 3A).

To provide DHBV proteins, we used helper plasmid pMD4 (1) in which the redundant DHBV genome is under control of a metallothionein promoter and lacks part of the 5' encapsidation signal ϵ which renders it encapsidation deficient (see Fig. 2) (21). In pMD5, the 3' ϵ and DR1 regions (nt 2526 to 2662) were deleted in addition (see Fig. 3A). Thereby, most of the terminal redundancy was destroyed. In the DHBV16/DBHV3 chimeric helper pMD5-D3, the pre-S region from nt 832 to 1294 was replaced by the analogous region of DHBV3 (see Fig. 3A). To avoid contamination of the plasmid preparations with other DHBV-containing constructs, all plasmids were prepared in a laboratory in which no DHBV constructs were used. To check purity, we tested the transfer plasmid preparations by PCR, which specifically amplifies the DHBV S gene, and infected duck hepatocytes with the medium of LMH cells solely transfected with the transfer plasmid.

LMH packaging cell line. The LMH packaging cell line was produced essentially as described previously (8). LMH chicken hepatoma cells (5) were transfected with plasmid pMD5 along with plasmid pSV2Neo conferring Geneticin resistance. Cells were maintained in Dulbecco's minimal essential medium-F12 with 10% fetal calf serum, 250 IU of penicillin-streptomycin, 1 mM sodium pyruvate, 1 mM nonessential amino acids, and 1 mM glutamine. Geneticin (200 μ g/ml; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added from day 1 posttransfection. Geneticin-resistant cell clones were isolated by using cloning rings and were seeded with Geneticin-sensitive LMH cells to support the growth of transfected clones. The mixed culture was grown in medium without Geneticin for 2 days, and Geneticin (200 μ g/ml) was then added to remove helper cells. Resistant clones were further expanded.

Production of recombinant virus stocks. For the production of recombinant DHBV (rDHBV), LMH cells were cotransfected at 30 to 40% confluence with 25 μ g of the respective transfer construct and 12 μ g of the helper construct per 15-cm-diameter dish by using the calcium-phosphate method. Cell culture medium containing recombinant virus was collected from days 2 to 6 posttransfection. Virus stocks were concentrated 10-fold by precipitation with polyethylene glycol and quantified following cesium chloride gradient centrifugation to identify enveloped virions. Virus titers were measured as DNA-containing enveloped viral particles (v.p.) determined by dot blot hybridization of the gradient fractions relative to a DHBV DNA standard as described before (21).

Isolation and infection of PDHs. PDH cultures were isolated by standard two-step collagenase perfusion and subsequent differential centrifugation as described before (14). Livers from 2- to 3-week-old ducklings were perfused via the portal vein. From the obtained liver cell suspensions, hepatocytes were sedimented at 50 $\times$ g, washed three times, and seeded at a density of 10^6 cells per ml (2×10^5 cells/cm²). Cells were maintained at 37°C in 5% CO₂ in supplemented Williams E medium (50 μ g of gentamicin/ml, 50 μ g of streptomycin/ml, 50 IU of penicillin/ml, 2.25 mM L-glutamine, 0.06% glucose, 23 mM HEPES [pH 7.4], 4.8

μ g of hydrocortisone/ml, 1 μ g of inosine/ml, 1.5% dimethyl sulfoxide) without the addition of serum (supplements were obtained from Sigma Aldrich).

Two days after plating, PDHs were infected overnight. As an inoculum, we used wild-type DHBV from a DHBV16-positive duck serum at a multiplicity of infection (MOI) of 10^1 to 10^{-7} v.p. per cell, rDHBV-GFP at an MOI of 100 v.p. per cell (unless otherwise indicated), the medium of transfected LMH cells, or the medium of the LMH packaging cell line.

Infection of ducklings. Pekin ducklings were obtained from a commercial supplier (Fa. Wichmann, Lastrup, Germany) directly after hatching. The day posthatching, sera were taken and 10 μ l was spotted onto a nitrocellulose membrane and analyzed for DHBV by using a ³²P-labeled DHBV DNA probe for detection. At day 2, the animals were infected by injecting a 200- μ l inoculum into a foot vein. Animals were kept in a specialized facility according to good laboratory practice guidelines, bled weekly, and sacrificed to obtain liver tissue at day 28 postinfection (p.i.) (except one animal sacrificed at day 21 p.i.).

Analysis of DHBV infection. Cell monolayers were fixed at day 4, 8, or 11 p.i. with paraformaldehyde at room temperature for 20 min. GFP expression was monitored by fluorescence microscopy with a standard fluorescein isothiocyanate filter set with excitation by blue light (488 nm). The number of wild-type DHBV-infected cells was determined by immunofluorescence staining of intracellular viral antigens by using polyclonal rabbit antisera against the DHBV core protein and the pre-S region of the DHBV large envelope (L) protein and a red fluorescent secondary goat anti-rabbit immunoglobulin G antibody (Alexa 568 nm; Molecular Probes, Leiden, The Netherlands) as described before (21). To detect DHBV replication, intracellular DNA was extracted from infected cells or duck livers, separated on a 0.8% agarose gel, and analyzed by Southern blotting with a ³²P-labeled DHBV DNA probe for detection as described previously (14). To detect in vivo transduction, a PCR was performed with a 5' primer hybridizing in the DHBV genome and a 3' primer hybridizing in the transgene. In addition, liver lysates were prepared, separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis as described (14), and analyzed for the DHBV S protein by Western blotting with monoclonal mouse antibody 7C.12 (kindly provided by J. C. Pugh) and an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G antibody. Protein bands were visualized by using the enhanced chemiluminescence system (Amersham, Cleveland, Ohio).

RESULTS

Recombinant DHBV stocks contain wild-type virus. To test whether rDHBV stocks contain contaminating wild-type DHBV, we used an rDHBV in which the S gene was replaced by GFP (rDHBV-GFP) (21). rDHBV-GFP is replication deficient due to the lack of the viral polymerase/reverse transcriptase. When used to infect PDHs, the number of GFP-expressing cells was proportional to the number of rDHBV particles in the inoculum (Fig. 1a).

In rDHBV-GFP, no envelope proteins are expressed (21) (schematic depiction in Fig. 2). Thus, the production of wild-type virus was detected by measuring the expression of envelope proteins by use of immunofluorescence staining against the DHBV L protein.

A sensitive assay for identifying replication-competent virus is the infection of duck hepatocyte cultures which support virus spread. Initial experiments confirmed that 10 to 100 enveloped DNA-containing v.p. from duck serum were sufficient to infect the hepatocyte cultures and to allow detection of replicating DHBV by immunostaining of foci of infected cells when multiple rounds of infection were allowed. The sensitivity of detection varied between different primary cell preparations and had to be determined for each infection assay separately. Therefore, all infection experiments were performed in triplicate with the same set of primary hepatocytes. When cells were fixed at a late time point to gain maximal sensitivity (Table 1), the number of foci caused by spreading virus was not directly proportional to the number of wild-type viruses in the inoculum, unlike experiments with replication-deficient rDHBV

FIG. 1. Detection of wild-type DHBV in rDHBV stocks prepared by cotransfection of hepatoma cells. (a) PDH cultures were infected with rDHBV-GFP at various MOIs. GFP expression is shown at day 6 p.i. resulting from infection at an MOI of 6, 25, and 100 v.p. per cell for 12 h. Magnification, $\times 200$. (b) PDH cultures were infected overnight with rDHBV-GFP at an MOI of 30 v.p./cell. Using fluorescence microscopy, rDHBV-GFP-infected cells were detected by GFP expression (left panel) at day 8 p.i. Subsequently, cells were fixed and stained for the DHBV L protein (middle and right panels) at days 8 and 11 p.i., respectively. Magnification, $\times 200$.

(Fig. 1a). At higher concentrations, foci get confluent. In addition to typical foci, single positive cells as well as small groups of DHBV-positive cells were detected. This was most probably due to the fact that secondary foci evolve and allow interpretation only within a narrow range. To determine the cutoff of infection by end-term dilution, however, the assay proved very useful.

To detect wild-type virus in our rDHBV stocks, PDHs were infected with rDHBV-GFP stocks prepared under stringent conditions preventing accidental cotransfection of wild-type plasmids. All plasmids used for the production of recombinant virus stocks were prepared in a separate laboratory by using separate solutions, single-use plastic material, and filtered tips, and they were tested for purity by PCRs that selectively amplified the GFP gene, the DHBV S gene, or the GFP insert in the S gene. Furthermore, when the culture medium of pCD16-GFP-transfected LMH cells was used to infect PDHs, no wild-type DHBV was detected by immunofluorescence staining of DHBV proteins (data not shown).

Successful transduction by recombinant viruses was monitored by GFP expression. Following infection with rDHBV-GFP at an MOI of 30 v.p./cell, at day 8 p.i. single GFP-expressing cells were detected (Fig. 1b, left panel). Cells were fixed and stained for the DHBV L protein, which is not expressed by rDHBV-GFP. At day 8 p.i., the foci of 10 to 20 DHBV-positive hepatocytes were detected (Fig. 1b, middle

panel). At day 11 p.i., almost all hepatocytes stained positive for the DHBV L protein (Fig. 1b, right panel), whereas still only single cells expressed GFP. This result pointed to a presence of replicating wild-type virus in the rDHBV-GFP stock which had spread throughout the cell culture.

Modification of plasmid constructs used for the production of rDHBV. In principal, wild-type virus that emerged during recombinant virus production could result from homologous recombination between the plasmids used. The plasmid constructs we used for the production of rDHBV shared sequence homologies and contained redundant genomic sequences and thus allowed homologous recombination.

As depicted in Fig. 2, a single intermolecular recombination event between transfer plasmid pCD16-GFP and helper plasmid pMD4 would be sufficient to restore a functional encapsidation signal ϵ at the 5' end of the encapsidation-deficient helper and thus to reconstitute a replication-competent DHBV genome. In addition, oligomerization of helper plasmids by recombination between nt 2579 to 2845 of the DHBV genome would reconstitute a wild-type DHBV genome because the 3' terminal redundancy contained a second ϵ sequence (Fig. 2).

To minimize the chance of recombination, the DHBV genome in the helper and transfer plasmids was modified. First, the terminal redundancy in helper plasmid pMD4 was diminished by deleting the 3' end of the DHBV genome (nt 2526 to 2662) containing the direct repeat DR1 and the ϵ signal. With

FIG. 2. Reconstitution of a wild-type DHBV genome by homologous recombination events. Schematic presentation of a transfer plasmid (pCD16-GFP) and a helper plasmid (pMD4) used for the production of rDHBV. Both plasmids contain a terminally redundant DHBV16 genome (thick black line, numbers represent DHBV nucleotides) under the control of a foreign promoter (cytomegalovirus [CMV] or metallothionein [MT], open arrows). The redundancy spans the DHBV *cis* signals (grey open boxes) for RNA encapsidation (ϵ) and reverse transcription (DRI [1] and DRII [2]) and the DHBV polyadenylation signal (A_n , depicted by a diamond). Transcription starts are marked by closed arrows. In the transfer plasmid pCD16-GFP, the GFP transgene is represented by a white open box. In the helper plasmid pMD4, the 5' ϵ signal is deleted ($\Delta\epsilon$), which renders the transcribed RNA encapsidation deficient. Grey zones mark the viral sequences in which recombination has to occur to reconstitute a wild-type DHBV genome. Homologous recombination either between helper and transfer plasmids (helper $\times$ transfer, upper panel) or in the terminal redundancies of two helper plasmids (helper $\times$ helper, lower panel) can reconstitute a DHBV genome which allows transcription of wild-type pg RNA (depicted by a wavy line). DHBV ORFs are depicted by open boxes (core, pre-S, S, and polymerase).

the resulting helper plasmid, pMD5 (Fig. 3A), the ability to generate a replication-competent DHBV genome by oligomerization was completely abolished.

Second, transfer plasmid pCD16-GFP was modified. The pg

TABLE 1. Detection of low numbers of wild-type DHBV by the detection of foci of infected duck hepatocytes by immunofluorescence staining of the DHBV L protein^a

No. of WT DHBV (v.p.)	No. of infected cells	No. of foci of DHBV-positive hepatocytes (triplicate infections)
10 ²	10 ⁶	2, 0, 0
10 ³	10 ⁶	2, 3, 0
10 ⁴	10 ⁶	10, 12, ND
10 ⁵	10 ⁶	>80, >80, >80

^a PDHs were infected with the indicated number of serum-derived wild-type (WT) DHBV16 determined to be enveloped DNA-containing virus particles (see Materials and Methods). The number of foci of cells infected with replicating wild-type virus was determined at day 21 p.i. by immunofluorescence staining for the DHBV L protein. ND, not determined.

RNA transcribed from the DHBV genome of pCD16-GFP had, due to the insertion of the GFP gene, an overlength of 175 nt in comparison to a DHBV wild-type pg RNA. A 173-nt fragment between DHBV nt 1847 and 2020 in the transfer plasmid was deleted to compensate for a potential replication disadvantage due to genome overlength. In addition, stop codons were introduced into all remaining viral ORFs. The resulting plasmid, pCD16-GFP-2 (Fig. 3A), transcribed RNAs of the wild-type length from which only GFP was expressed.

Using the modified plasmids for virus production, two distinct recombination events between helper and transfer plasmids at the 5' end (between DHBV nt 2579 and 2660) and at the 3' end (between DHBV nt 2020 and 2526) are required to reconstitute a replication-competent DHBV genome (Fig. 3A).

Wild-type virus is generated by recombination. To investigate whether wild-type virus was generated by recombination, a chimeric helper containing sequences of DHBV16 and

FIG. 3. Characterization of replicating wild-type virus from rDHBV stocks. (A) Schematic presentation of modified plasmids for the production of rDHBV. In pCD16-GFP-2, a 173-nt region (marked with V) was deleted. Additionally, stop codons (marked with X) were introduced 5' of the viral ORFs (nt 2660 [core], nt 183 [polymerase], and nt 823 and 1165 [L]). In helper plasmid pMD5, most of the terminal redundancy was eliminated by deletion of the 3' DR1 (1) and ϵ sequences (ϵ); DR2 is marked by the number 2. Using pCD16-GFP-2 and pMD5, two distinct recombination events (grey zones) were required to generate replication-competent DHBV. To trace homologous recombination, a subtype-chimeric helper plasmid pMD5-D3 was constructed in which the DHBV16 pre-S region was replaced by a DHBV3 sequence which lacks one *HindIII* restriction site. A_n , DHBV polyadenylation site. (B) Duck hepatocytes were infected with rDHBV produced by using either pMD5 or the subtype-chimeric pMD5-D3 as a helper. At day 20 p.i., DNA was isolated from cells infected with either virus and DHBV DNA was detected by Southern blot analysis with a ^{32}P -labeled DHBV DNA probe. Undigested DNA was applied to the left lane, *HindIII*-digested DNA from cells infected with virus produced with pMD5 was applied to the middle lane, and *HindIII*-digested DNA from cells infected with virus produced with chimeric helper pMD5-D3 was applied to the right lane. M, DNA size marker. The expected sizes of the rc, ccc, and ss DHBV DNAs are indicated as well as the sizes of the restriction fragments. The right panel represents a longer exposure of the same blot.

DHBV3 was constructed. The pre-S region of the DHBV16-based helper plasmid pMD5 was exchanged with the analogous DHBV3 sequence (pMD5-D3), which gives a different restriction pattern. If wild-type virus was reconstituted in LMH cells following cotransfection with pCD16-GFP-2, it had to contain the pre-S sequence of subtype D3 (Fig. 3A).

rDHBV stocks were produced by cotransfection of pCD16-GFP-2 with either pMD5 or pMD5-D3 and were used to infect PDHs. Replication-competent virus was expected to spread throughout the cell culture. At day 20 p.i., when viral spread was expected to be complete, DNA was isolated from the cells and analyzed by Southern blotting after digestion with the restriction enzyme *HindIII*. *HindIII* cuts circular DHBV16 DNA into three fragments of 241, 1,079, and 1,701 bp in length. As the pre-S region of DHBV3 lacks a *HindIII* restriction site, circular chimeric DHBV16/DHBV3 DNA derived from homologous recombination can be identified by two fragments of 1,701 and 1,316 bp following *HindIII* digestion (Fig. 3A).

Southern blot analysis (Fig. 3B) of undigested DNA, isolated from the cells infected with rDHBV generated by cotransfection of pCD16-GFP-2 and pMD5 (marked pMD5) showed the presence of rc, linear, ccc, and single stranded (ss) DHBV DNAs (Fig. 3B, lane pMD5). This proved that DHBV had replicated in the cells. Restriction analysis with *HindIII* revealed three distinct bands corresponding to the expected

DNA fragments of DHBV16, whereby the smallest (241 nt) fragment was only detected after a longer exposure (Fig. 3B, right panel).

However, when rDHBV was produced by cotransfection of pCD16-GFP-2 and the chimeric helper pMD5-D3 and was then used as an inoculum, *HindIII* digestion of isolated DNA resulted in only two bands of 1.7 and 1.3 kb in size (Fig. 3B, lane pMD5-D3). This proved that the replicating virus contained the DHBV3 pre-S region, which had been generated following homologous recombination between transfer and chimeric helper plasmids and did not result from contaminating plasmids. We therefore concluded that the replicating virus contained in the rDHBV stocks arose from recombination between plasmids, most probably from homologous recombination.

Establishment of a stable packaging cell line for rDHBV production. In order to reduce the chance of homologous recombination, a packaging cell line was generated by stable integration of plasmid pMD5 into LMH cells. Various clones, expressing the DHBV core and L proteins (as determined by immunofluorescence staining), were tested for their helper function by transient transfection of transfer plasmid pCD16-GFP-2. The amount of rDHBV produced was analyzed in the cell culture medium by CsCl step gradient centrifugation and subsequent DNA dot blot analysis (Fig. 4A). In addition, cellular DNA was isolated and analyzed for the presence of in-

FIG. 4. Comparison of the LMH packaging cell line and cotransfection of helper and transfer plasmids used for the production of recombinant virus stocks. (A) Recombinant virus produced with the packaging cell line by transient transfection with pCD16-GFP-2 was subjected to CsCl step gradient centrifugation to separate naked core particles from enveloped virus particles. DNA dot blot analysis of gradient fractions collected from bottom to top shows that enveloped rDHBV is produced. (B) The amount of virus particles was determined by quantitation in comparison to a DHBV DNA standard. Virus titers (averages $\pm$ standard deviations) determined in four independent experiments are shown. The LMH packaging cell line was transfected with different transfer plasmids. Alternatively, LMH cells were cotransfected either with pMD4 and pCD16-GFP or pCD16-GFP-2 or with pMD5 and pCD16-GFP or pCD16-GFP-2.

tegrated DHBV sequences (data not shown). One clone was selected as a packaging cell line which carried a single integrate and was able to reproducibly produce recombinant virus stocks at titers up to 5×10^8 v.p. per ml.

In the following, we measured the yield of recombinant virus generated with various plasmids and the packaging cell line in parallel. Since regulatory elements in the DHBV genome necessary for circularization and reverse transcription are not fully characterized and may have been affected by the mutations or deletions introduced into the transfer plasmid, we also used pCD16-GFP or pCD16-GFP-2 in parallel. As shown in Fig. 4B, no highly significant difference in virus yield was noticed in four independent experiments, neither between the different

TABLE 2. Quantitation of replicating wild-type DHBV contained in rDHBV stocks produced by cotransfection of helper plasmid pMD4 or pMD5 or with an LMH packaging cell line

Plasmid or cell line	Total no. of rDHBV in inoculum (v.p.) ^a	No. of cells infected ^b	No. of foci of infected cells ^c	WT DHBV/rDHBV ratio ^d
Helper plasmids				
pMD4 with:				
pCD16-GFP ^e	10 ⁸	10 ⁶	222 $\pm$ 52.5	5 $\times$ 10 ⁻⁶
pCD16-GFP-2 ^e	10 ⁸	10 ⁶	205.0 $\pm$ 27.6	4 $\times$ 10 ⁻⁶
pMD5 with:				
pCD16-GFP ^e	10 ⁸	10 ⁶	27.5 $\pm$ 12.0	7 $\times$ 10 ⁻⁷
pCD16-GFP-2 ^e	10 ⁸	10 ⁶	5 $\pm$ 2.6	3 $\times$ 10 ⁻⁷
Packaging cell line	5 $\times$ 10 ⁸	5 $\times$ 10 ⁶	0 $\pm$ 0	<2 $\times$ 10 ⁻⁸

^a Triplicate infection of 10⁶ PDHs infected with the indicated virus stock. The number of rDHBV is given as DNA-containing enveloped viral particles.

^b Number of hepatocytes infected in each experiment.

^c The number of foci of cells infected with wild-type virus was determined at day 8 p.i. by immunofluorescence staining for the DHBV L protein, which is not expressed by the recombinant viruses. The means and standard deviations of three independent experiments are shown.

^d The number of replicating wild-type (WT) DHBV in the respective rDHBV stock was calculated from the number of foci of infected cells. The detection limit of the assay shown was determined to be 2×10^{-8} by using serum-derived wild-type DHBV. In none of the infection experiments with rDHBV generated with the help of the packaging cell line was a focus of infected cells detected.

^e Transfer plasmid used for rDHBV production.

plasmid constructs nor when the packaging cell line was used ($P > 0.01$). However, the virus yields showed a high interexperimental variance, and the average yield varied between 2×10^7 and 5×10^8 v.p./ml.

Use of a packaging cell line to avoid recombination between plasmids. In order to determine the proportion of wild-type virus, primary hepatocytes were infected at an MOI of 100 v.p./cell with rDHBV stocks produced with either of the two different helper plasmids or with the packaging cell line. To compare the infectivity and transduction capacity of each virus stock, we determined the amount of GFP-expressing cells at day 8 p.i. in living cells (data not shown). The numbers of GFP-expressing cells were comparable, indicating similar infectivity of all used rDHBV stocks.

To detect replicating virus, cells were subsequently fixed and stained for viral envelope proteins with a red fluorescent secondary antibody. At this time point, progeny virus had been released into the cell culture medium and spread to neighboring cells. Due to this spread, the foci of 10 to 20 hepatocytes infected with wild-type DHBV could be detected by immunofluorescence staining for the DHBV S protein (Fig. 1b, middle panel). The proportion of replicating wild-type virus in the different rDHBV stocks tested is given in Table 2.

When rDHBV stocks produced with helper pMD4 were used for infection, relatively high numbers of foci of cells infected with wild-type virus were detected. By parallel infections with DHBV positive duck serum, we determined the numbers of replicating focus-forming viruses in rDHBV stocks (data not shown). Assuming that wild-type DHBV particles are equally infectious, we determined that 10⁵ rDHBV produced by cotransfection of pCD16-GFP and pMD4 contained one replicating wild-type virus particle.

As shown in Table 2, the use of the modified transfer plas-

FIG. 5. Replication of DHBV *in vivo* following infection with rDHBV. Ducklings were infected at day 2 after hatching with rDHBV produced either by cotransfection or by using the LMH packaging cell line. The amount of rDHBV used is indicated. The animals were sacrificed at day 28 p.i., and DHBV replication was detected by Southern blot analysis of total liver DNA by using a ³²P-labeled DHBV DNA probe (upper panel). Western blot analysis of liver lysates detected the DHBV S protein in all animals that replicated DHBV (lower panel). wt, wild type.

mid pCD16-GFP-2, which required two independent recombination events to generate replication-competent virus, had very little effect. When helper pMD5 was used, which abolished the possibility that replication-competent virus resulted from oligomerization of helper plasmids, the amount of focus-forming, replicating wild-type viruses was reduced by a factor of 10 (Table 2). From this we concluded that helper-helper recombination within the terminal redundancy had a high impact on reconstitution of wild-type DHBV.

However, when the packaging cell line was used for rDHBV production, replicating virus was no longer detected (Table 2). This result was confirmed by numerous other infection experiments which used rDHBV stocks prepared with the packaging cell line in which the observation time was prolonged up to 20 days (data not shown). We therefore concluded that the generation of wild-type virus can be avoided by using a packaging cell line.

Infection of ducklings with rDHBV stocks from different sources. For gene therapeutic purposes, it is important to know whether replicating virus contained in the recombinant virus stocks may cause productive DHBV infection *in vivo*. Therefore, 7 ducklings were infected with rDHBV produced by cotransfection, and 5 animals were infected with recombinant virus produced with the packaging cell line. rDHBV (2.5×10^8 to 1×10^9 v.p. per animal) was injected intravenously as indicated on Fig. 5.

The transduction efficiency of the rDHBV stocks was monitored by PCR analysis of DNA extracted from liver lysates of the infected animals. Transgene cDNA was amplified from the livers of all animals tested (data not shown).

To identify wild-type DHBV, Southern and Western blot analyses were conducted on liver extracts to detect DHBV replicative intermediates and expression of S protein. As shown in Fig. 5, DHBV replicative intermediates were detected in the animals which had received rDHBV produced by

cotransfection, except in the two animals which had received the lowest dose of rDHBV (2.5×10^8 v.p.). In the livers of all animals in which DHBV replicated, the DHBV S protein, which is not expressed by the rDHBV used, was detected. This showed that the rDHBV stocks produced by plasmid cotransfection contained wild-type DHBV which is infectious *in vivo*.

However, neither DHBV rc or ss DNA nor S protein was detected in the livers of the 5 animals infected with rDHBV produced with the packaging cell line, 4 of which had received high titers of rDHBV (10^9 v.p.). Furthermore, sera from these animals did not contain infectious virus, as determined by their inability to infect PDH cultures (data not shown).

To determine the amount of wild-type virus necessary to establish a productive DHBV infection in the animal, 3 ducklings were infected with each of the following amounts of a DHBV16-positive duck serum: 10^5 , 10^4 , 10^3 , and 10^2 v.p. By the assays described, we determined one animal inoculated with 10^5 v.p. and one animal inoculated with 10^4 v.p. to be productively infected (data not shown).

Taken together, these results show that the replicating wild-type virus present in the recombinant virus stocks produced by standard cotransfection is sufficient to induce a productive hepadnaviral infection *in vivo*. No replication-competent virus was detected, however, when rDHBV stocks were produced with the help of a packaging cell line.

DISCUSSION

The data presented demonstrate that recombinant hepadnavirus stocks may contain significant amounts of wild-type or replication-competent virus when produced by transfection of plasmids which contain redundant viral genomes or share homologous sequences. The coproduced wild-type virus may alter the experimental results when viral mutants are analyzed. If recombinant HBV shall serve as a viral vector for gene therapy (7, 21), it is a prerequisite that the vector stocks are free of replication-competent virus.

We propose homologous DNA recombination between transfected plasmids to be the origin of the replicating virus observed. Recombination occurred either between transfer and helper plasmids sharing homologous sequences or in between redundant sequences on the same helper plasmid. Once a wild-type genome has been generated, it will be further amplified within the cell because it can be reimported into the nucleus by the viral capsid to serve as an additional transcription template.

Consequently, deletion of redundant viral sequences in the helper plasmid and the generation of a packaging cell line with this plasmid proved sufficient to reduce accidental coproduction of replication-competent virus to an undetectable level. Nevertheless, titers of the recombinant viruses obtained remained at the same level whether produced with original or modified helper and transfer plasmids or with the packaging cell line.

The fact that we were able to knock out all remaining DHBV ORFs in the transfer plasmid without reduction of the recombinant virus titers indicates that all DHBV proteins can be provided *in trans*. In particular, the core protein, which is expressed by the original but not by the modified constructs, has no obvious *cis*-acting function during viral morphogenesis.

Infection of primary hepatocytes proved to be the most-sensitive and robust assay for detecting infectious DHBV. We were able to detect infection and viral spread following inoculation with as few as 10 enveloped v.p. per ml. By repeated infection with the same serum-derived DHBV as an inoculum, however, we found variability between different primary cell preparations.

These results indicated that a high proportion of enveloped DHBV particles is infectious. This confirmed similar results obtained *in vivo* by Jilbert et al. when Pekin ducklings were infected (11). In our hands, however, infection of ducklings with the very same virus stock could only be obtained when 10^4 v.p. were contained in the inoculum. Apparently, there is a broad variation in susceptibility between the different Pekin ducklings used by different groups.

The number of foci formed in cell culture following infection with replicating wild-type DHBV was, unlike that in infection experiments with replication-deficient rDHBV, not directly proportional to the number of viral particles in the inoculum. This was most probably due to a virus spread via the cell culture medium in addition to direct cell-to-cell spread.

It has been known for a long time that mammalian cultured somatic cells contain enzymatic machinery to efficiently mediate homologous recombination between transfected plasmid molecules (5, 6, 24, 27). Furthermore, when mutant DHBV genomes were coinjected directly into the livers of ducklings with subgenomic viral DNA fragments spanning the mutation, wild-type recombinants arose (26). In the transfected cell, homologous recombination can theoretically occur at different levels: (i) homologous sequences in the transfected plasmids are recombined at the DNA level, (ii) recombination is caused by a template switch of the cellular RNA polymerase II during transcription of viral pg RNA, and (iii) if more than one RNA was packaged into nucleocapsids, recombination could occur during reverse transcription by a template switch of the viral polymerase.

Sequence homology promotes recombination events on the DNA level and is an ideal precondition for a template switch of the cellular RNA polymerase II. Folger et al. (6) determined that, in cultured mammalian cells, homologous recombination between coinjected plasmids occurs within 1 h at a high frequency, whereas the frequency of homologous recombination between given chromosomal markers is much lower. They hypothesized that exogenous DNA assembles into nucleosomes, which exposes it to the cellular recombination machinery. This exactly fits our observation that homologous recombination led to the production of wild-type virus on a regular basis when homologous sequences were present in transfected plasmids, even when two distinct recombination events were necessary and when recombination in narrow genome regions was required. In contrast, recombination was not detected anymore when a packaging cell line was used.

Alternatively, recombination could be caused by a template switch of the cellular RNA polymerase II during transcription of hepadnaviral pg RNA. Chang and Taylor (4) found that mammalian RNA polymerase II can achieve intramolecular template switching during RNA-directed transcription of a discontinuous RNA template. Thus, one may also speculate about its capability to switch the template during DNA-di-

rected transcription. However, our experimental system did not allow us to obtain any further evidence along that line.

The hepadnaviral reverse transcriptase is a polymerase well known to perform several template switches during the viral replication cycle. Thus, it would be capable of synthesizing a recombined genome from different templates. Reverse transcription, however, takes place inside the icosahedral viral nucleocapsid (1), which provides very limited space, and it is not known whether two pg RNAs can be encapsidated.

Taken together, we think that recombination, most probably homologous recombination, of DNA is responsible for the coproduction of wild-type virus with recombinant hepadnaviruses. If mutant or recombinant hepadnaviruses are used in critical experiments, we would propose the use of a packaging cell line for the generation of virus stocks.

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