Efficient Duck Hepatitis B Virus Production by an Avian Liver Tumor Cell Line

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Duck hepatitis B virus (DHBV) is produced in small amounts following transfection of human hepatoma or hepatoblastoma cell lines with cloned viral DNA. In a search for better hosts for DHBV replication, two avian liver cell lines were investigated. One of these cell lines, LMH, produced 5 to 10 times more DNA replicative intermediates and 10 to 20 times more infectious DHBV than did either of the two human cell lines, HuH-7 and Hep G2. Utilization of cell lines in genetic analyses of virus replication is often dependent upon obtaining efficient complementation between cotransfected viral genomes. We assayed transcomplementation of a viral polymerase (*pol*) gene mutant, which is rather inefficient in transfected human cells, and found that viral DNA synthesis was at least 20 times more efficient following cotransfection of LMH cells than in similarly transfected HuH-7 cells. Recombination, a potential interpretation problem in complementation assays, occurred at low levels in the cotransfected cultures but was substantially reduced or eliminated by creation of an LMH subline stably expressing the viral polymerase. This cell line, *pol-7*, supported the replication of DHBV *pol* mutants at ca. 10 to 15% of the level of virus replication obtained following transfection with wild-type viral DNA. By transcomplementation of a *pol* gene mutant in LMH cells, we were able to produce sufficient virus with the mutant genome to investigate the role of polymerase in covalently closed circular DNA amplification. Our results substantiate the hypothesis that covalently closed circular DNA is synthesized by the viral reverse transcriptase.

Research on hepadnavirus gene expression has, until recently, depended upon the introduction of viral DNAs into cell lines that were resistant to virus infection and incapable of supporting virus replication from transfected DNAs. The lack of virus replication was probably due, in part, to inactivity of the viral core promoter in the cells that were investigated. This promoter directs transcription of the viral pregenome, the template for synthesis of viral minus-strand DNA, which occurs through reverse transcription. Two sets of developments over the past 4 years have changed this situation. First, Tuttleman et al. (45) demonstrated that primary cultures of duck hepatocytes synthesize progeny virus following in vitro infection with duck hepatitis B virus (DHBV), Gripon et al. (11), Rijntjes et al. (30), and Ochiya et al. (25) showed that human hepatitis B virus (HBV) infects and replicates in primary human hepatocyte cultures, and Aldrich et al. (3) found that primary woodchuck hepatocytes were susceptible both to woodchuck hepatitis virus (WHV) and to ground squirrel hepatitis virus. Second, permanent cell lines have been identified that, although resistant to hepadnavirus infection, nevertheless produce infectious virus following stable or transient transfection with cloned viral DNAs. HBV production has been obtained following transfection of the human liver tumor lines Hep G2, HuH-6, and HuH-7 (1, 6, 36, 43, 44, 48) or the Buffalo rat liver line Q7 (38) and, with a heterologous (metallothionein) promoter directing pregenome synthesis, in transfected Hep G2 and HeLa cells (14). WHV production has been reported following transfection of Hep G2 cells and, when pregenomic RNA synthesis is directed by heterologous (human cytomegalovirus immediate early [CMV-IE]) promoter rather than the endogenous WHV promoter, of NIH 3T3 cells and turkey

embryo fibroblast cultures (33, 35, 49). Finally, DHBV is synthesized in both HuH-7 and Hep G2 cells transfected with cloned DHBV DNA (12, 28).

The importance of these developments is seen in the demonstration that it is possible to carry out a mutational analysis of hepadnavirus genomes (32), without resorting to animal testing, to determine the effect of mutations on production of infectious viruses from the transfected cell lines (7, 34). A potential limitation in using the Hep G2 or HuH-7 cell lines in a genetic analysis of DHBV replication lies in the fact that these cells produce very small amounts of virus in a transient-transfection assay, even when a wild-type genome is used. For example, Pugh et al. (28) estimated that transfected HuH-7 cells produced ca. 0.3% the amount of virus found in the serum of a congenitally infected duckling. With this amount of virus production, it is technically difficult to perform an in vitro study of the consequences to viral infectivity of mutations that severely diminish, but do not eliminate, infectious particle production and to investigate early steps in infection by mutated or wild-type virus produced by the transfected cells (e.g., conversion of the virion relaxed-circular [RC] genome to the covalently closed circular [CCC] DNA template for viral RNA synthesis).

During the course of our analyses of the DHBV genome, we therefore decided to investigate two additional cell lines of hepatocellular origin that we believed, a priori, might support DHBV production at a higher level. Unlike the cell lines described above, both were of avian origin. In addition, both expressed a number of markers of differential hepatocytes, such as apoVLDLII and apoE (4). The LMH cell line is derived from a liver tumor that arose in a chicken treated with diethylnitrosamine (15); the IQ1A cell line is derived from a liver tumor that developed in a Japanese quail

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injected with the avian retrovirus MC-29 (M. Linial, personal communication). The quail line retains the MC-29 genome, but not the helper virus, and therefore does not produce infectious MC-29 virus. Kawaguchi et al. (15) reported that the LMH cells also fail to produce any retroviruses, as determined in an assay of the tissue culture medium for reverse transcriptase-containing particles.

We report here that LMH cells transfected with constructs containing either a tandem DHBV dimer (pSP.DHBV5.1Galx2) or a pregenomic-length DHBV insert with transcription directed by the CMV-IE promoter (pCMVDHBV) produced 20 times more infectious DHBV and contained 5- to 10-fold-higher levels of viral DNA replicative intermediates than did IQ1A, Hep G2, or HuH-7 cells, which served as controls. The opposite result was obtained when Hep G2, HuH-7, and LMH cells were transfected with pCMW82, a plasmid similar to pCMVDHBV but containing a WHV insert (35), suggesting that the selective improvement in DHBV DNA synthesis in LMH cells was not due simply to the efficiency of the promoter controlling pregenome transcription. The high levels of virus production by LMH cells correlated with efficient *trans*-complementation following cotransfection by mutated viral genomes containing alterations of different genes. In addition, we obtained evidence suggestive of low levels of recombination between cotransfected genomes in both the LMH and HuH-7 cell lines. The problem of recombination was apparently reduced, at least in the case of viral *pol* gene mutations, by construction of an LMH cell line stably transformed with only one copy of the DHBV polymerase gene.

MATERIALS AND METHODS

Plasmid DNA. Pugh et al. (28) demonstrated that transfection of HuH-7 cells with the plasmid pSP.DHBV5.2Galx2, in which transcription of viral mRNAs was under the control of internal viral sequences, resulted in infectious virus production. pSP.DHBV5.2Galx2 contains a tandem dimer insert of DHBV in the *EcoRI* site of the pSP65 multiple cloning site oriented so that nucleotide (nt) 1 in the DHBV sequence is adjacent to pSP65 nt 11. To facilitate further constructions, the DHBV dimer insert was recloned in the reverse orientation in the same *EcoRI* site of pSP65. This plasmid is called pSP.DHBV5.1Galx2, and DHBV transcription is still controlled by DHBV sequences (Fig. 1A).

The plasmid pCMVDHBV places transcription of the RNA pregenome of DHBV under the control of the CMV-IE promoter. To construct this clone, pSP.DHBV5.1Galx2 was linearized with *SalI* (in the pSP65 polylinker) and partially digested with *AflII* (DHBV nt 2526 [20]) to yield a DHBV fragment of greater than unit length. The vector pBC12/CMV/IL-2 (8) was modified to yield the clone pTTW-1, in which a 43-nt polylinker [containing the *SacI*, *BclI*, *AflII*, *HindIII*, *BamHI*, *KpnI*, *SalI*, and *SmaI* restriction sites (5' to 3')] was inserted in place of a *SacI* (nt 663)–*SmaI* (nt 2142) restriction fragment. The DHBV *AflII*–*SalI* fragment was then inserted into this polylinker to place the DHBV pregenome cap site (DHBV nt 2529) in the position of the CMV-IE cap site (Fig. 1B). Since precore translation initiates upstream of nt 2526, this construct does not encode the precore protein; however, this protein is nonessential for DHBV replication (6, 32). With this construction, all viral transcripts should still terminate at the viral polyadenylation site at DHBV nt 2772.

A plasmid designated pCMVpol which was capable of expressing the DHBV polymerase gene was also con-

FIG. 1. Schematic representation of the plasmids used in these studies. Lines denote DHBV sequences; boxes denote vector or control sequences. Numbers refer to nucleotides in the DHBV genome, using the numbering scheme of Mandart et al. (20) such that nucleotide 1 is at the unique *EcoRI* site in the DHBV genome. (A) pSP.DHBV5.1Galx2. This plasmid contains a head-to-tail dimer of the DHBV genome inserted into the *EcoRI* site of the pSP65 multiple cloning site such that a full-length pregenomic RNA can be transcribed from the DHBV core promoter. All restriction cleavage sites in the DHBV genome which were used in the construction of various subclones are indicated. The *AflII* site at nt 2526 is located immediately upstream of the normal start site of transcription from the DHBV core promoter. The *HindIII* site at nt 14 is located upstream of the polymerase open reading frame. (B) pCMVDHBV. Transcription of the DHBV pregenome is placed under the control of the human CMV-IE region. The 3.5-kbp *AflII*–*SalI* DHBV fragment was subcloned into the plasmid pTTW-1 (described fully in the text). Symbols: ▨, CMV-IE region; →, CMV promoter containing DNA sequences. (C) pCMVpol. This is a DHBV polymerase expression plasmid in which control of transcription of an mRNA encoding the DHBV polymerase gene lies with the CMV-IE promoter. This was constructed by deleting the 0.5 kbp *AflII*–*HindIII* fragment from the pCMVDHBV clone. (D) pCMVNP1-2. Into the *SalI* site downstream of the DHBV polymerase gene in pCMVpol, a 1.8-kbp neomycin resistance cassette has been inserted (—). In this cassette, the murine β -globin promoter is used to drive transcription of the neomycin resistance gene and polyadenylation is encoded by the simian virus 40 early polyadenylation signal. This subclone was used to generate a stably transformed LMH cell subline which produces a functional DHBV polymerase activity. (E) pCMV Δ Ppol. This plasmid contains the same DHBV polymerase open reading frame as pCMVpol, but the TATA box of the CMV-IE promoter contained in a 0.2-kbp fragment between the *NcoI* and *HindIII* sites of the CMV-IE region is deleted. Therefore, although a large proportion of the CMV-IE enhancer remains, no characterized promoter elements are present to promote transcription of a DHBV polymerase mRNA. The *NcoI* site in parentheses is no longer intact (see text).

structed. For this purpose, pTTW-1 was digested with *SalI* and *HindIII*, both of which cleave in the inserted polylinker immediately downstream of the CMV-IE promoter. A DHBV fragment generated by *HindIII* (DHBV nt 14) and *SalI* cleavage (in the polylinker) of pCMVDHBV was then

inserted (Fig. 1C). The vector pCMVpol was further modified for use in stable transformation: a cassette containing the neomycin resistance gene driven by the murine β -globin promoter and using the simian virus 40 early polyadenylation signal was cloned into the *SalI* site of pCMVpol such that transcription of both polymerase and neomycin resistance genes was in the same orientation. This plasmid is termed pCMVPN1-2 (Fig. 1D). For recombination analyses, a DHBV polymerase construct was generated which lacked the CMV-IE promoter element (termed pCMV Δ Ppol [Fig. 1E]). To make this plasmid, a restriction fragment ranging from the *NcoI* site (nt 455 in the CMV-IE promoter numbered by the method of Cullen [8]) to the *AflIII* site (including the TATA box and the CMV-IE promoter cap site) was removed from pTTW-1 to inactivate the promoter. The remaining vector fragment was then ligated following a repair reaction with the Klenow fragment of *Escherichia coli* DNA polymerase I. This intermediate construct was next cleaved in the remaining polylinker with *HindIII* and *SalI*, and the same *HindIII*-*SalI* fragment containing the polymerase open reading frame which was used in the generation of pCMVpol was inserted to create pCMV Δ Ppol.

For complementation assays, we also used a DHBV polymerase mutant plasmid provided by Jesse Summers, termed pSP.DHBV5.1RV718 (13), which contains a tandem DHBV dimer in pSP65, with a 2-base deletion at each *EcoRV* site at position 720. Therefore, no functional DHBV polymerase can be made in cells transfected with this plasmid, and DHBV DNA synthesis does not occur.

The construct pCMW82 (provided by Christoph Seeger), encoding wild-type WHV, has been described previously (35) and contains a WHV insert of greater than unit length with pregenome transcription directed by the CMV-IE promoter.

Cell cultures. Four established liver tumor cell lines were used: LMH, IQ1a, HuH-7, and Hep G2. Both avian cell lines (LMH and IQ1a) retain a number of markers for differentiated hepatocytes, although each line was derived from a distinct source (4). The LMH cells were initially grown in Waymouth medium containing 10% fetal calf serum, and this cell line has been adapted to growth in 1:1 Dulbecco minimal essential medium-Ham nutrient mixture F12 supplemented with 10 mM sodium bicarbonate and 10% calf serum. The IQ1a cell line was maintained in Ham nutrient mixture F10 supplemented with 10 mM sodium bicarbonate, 10% calf serum, 2% chicken serum (preheated at 56°C for 1 h), and 10% tryptose phosphate broth. Both LMH and IQ1a cell lines were passaged every 4 to 5 days at a 1:3 dilution. The LMH cells were not allowed to grow to heavy densities, because they became resistant to trypsinization and failed to form monolayers when passaged.

The mammalian cell lines (HuH-7 and Hep G2) have been described in some detail previously (2, 16, 17, 24). The HuH-7 cells were maintained in Dulbecco minimal essential medium-Ham nutrient mixture F12 plus 10% fetal calf serum, and the Hep G2 cells were maintained in minimal essential medium containing 10% fetal calf serum. These mammalian cell lines were passaged as above, and all four cell lines were incubated at 37°C in a CO₂ incubator with daily medium changes.

Primary hepatocyte cultures from 2-week-old Pekin ducklings negative for DHBV infection were prepared and maintained in serum-free medium as previously described (26, 27, 45). The cultures were infected the next day with either clarified medium from transfected cultures or pooled DHBV-positive serum. Following a 16- to 24-h adsorption period at

37°C, cultures were maintained in nutrient medium containing 100 μ g of suramin per ml with or without 1 mM phosphonoformic acid (PFA) as indicated. At 3 or 8 days postinfection, cultures were rinsed twice with PBS (150 mM NaCl, 7.2 mM Na₂HPO₄, 2.8 mM KH₂PO₄) prior to DNA extraction.

DNA transfection. All plasmid DNAs used in transfection assays were harvested from *E. coli* HB101 by the alkaline lysis method (21) followed by isopycnic centrifugation in cesium chloride gradients.

For transient transfection, cells were incubated on 60-mm-diameter tissue culture dishes for 18 to 24 h to 70 to 80% confluency. The actual number of cells that were seeded to produce this degree of confluency depended on the particular cell line. LMH and IQ1a cells were plated at 3.2×10^6 to 3.4×10^6 cells per dish, HuH-7 cells were plated at 1.65×10^6 to 1.8×10^6 cells per dish, and Hep G2 cells were plated at 2.5×10^6 to 3×10^6 cells per dish. At 2 h prior to transfection, 3 ml of fresh medium was placed on the cells. Although the LMH cells were initially grown in medium supplemented with 10% calf serum, for transfection the medium contained 10% fetal calf serum. Cultures were transfected with an amount of plasmid DNA (10 μ g) which gave the highest transfection efficiency, by using the calcium phosphate precipitation method of Graham and van der Eb (10), except that the precipitate was left on the cells for 14 to 16 h at 37°C. The medium was then changed daily until harvest, at which time the medium was clarified of floating cells and stored at -80°C. The monolayers were rinsed twice with PBS and either fixed with 95% ethanol-5% acetic acid at -20°C for fluorescence analysis of transfection efficiency with DHBV core-specific antisera or used for intracellular core particle DNA isolation.

For the generation of stable transformants, cultures were transfected as described above and, on day 2 posttransfection, passaged and changed to nutrient medium containing 200 μ g of geneticin (G418; Sigma Chemical Co.) per ml. Drug-resistant colonies were grown to approximately 0.3 cm in diameter and subsequently passaged as necessary in medium containing 100 μ g of G418 per ml, with medium changes on alternate days. After 10 passages (ca. 2.5 months), resistant clones were used in experiments.

Analysis of DNA. Viral DNAs were extracted from cytoplasmic core particles in transfected cultures essentially as described by Pugh et al. (28), except that the Hep G2 lysis buffer contained 1% Nonidet P-40 (equivalent to 10 times that necessary for lysis of LMH, IQ1a, or HuH-7 cultures).

Total nucleic acids, including nuclear and cytoplasmic viral DNAs, were extracted from DHBV-infected primary duck hepatocyte cultures by a 1-h incubation at 37°C in a solution containing 2 mg of pronase per ml, 0.1% sodium dodecyl sulfate, 0.15 M NaCl, 0.01 M Tris hydrochloride (pH 7.5), and 0.01 M EDTA, which was followed by a 1:1 phenol-chloroform extraction and precipitation with ethanol (26, 47). DNA from one quarter of each culture was subjected to electrophoresis in 1.5% agarose slab gels and transferred to nitrocellulose sheets (40, 46). CCC viral DNA was extracted as described by Wu et al. (47), subjected to electrophoresis through a 1.5% agarose gel, and analyzed by the method of Southern (40), essentially as modified by Wahl et al. (46). DHBV or WHV DNA was detected by hybridization with ³²P-labeled probes prepared from DNA clones of either DHBV or WHV.

DHBV virions were harvested essentially as described by Mason et al. (23). Briefly, virus in serum or in clarified media from transfected cells was layered on a step gradient con-

FIG. 2. Comparison of intracellular DHBV replicative intermediate DNA forms in transfected cultures of LMH, HuH-7, Hep G2, and IQ1a cells. Cytoplasmic core particle DNA was extracted from duplicate cultures of each cell line grown on a 60-mm dish at day 5 posttransfection with the DHBV dimer construct pSP.DHBV5.1Galx2. One quarter of this DNA was subjected to agarose gel electrophoresis for analysis. (A and B) The same gel containing DNAs from transfected LMH, HuH-7, and Hep G2 cultures. In panel A, the gel is stained with ethidium bromide, and in panel B, a Southern analysis following hybridization with a DHBV-specific DNA probe is shown. (C) DNAs from transfected LMH and IQ1a cultures were used for a Southern blot analysis. M, *Hind*III-digested bacteriophage λ DNA size markers and (in panel B) a 3-kbp DHBV DNA fragment; RC, 3-kbp RC DHBV DNA; DSL, double-stranded linear 3-kbp DHBV DNA; SS, single-stranded linear 3-kilobase DHBV DNA.

taining equal volumes of 10 and 20% (wt/vol) sucrose in 0.15 M NaCl–0.02 M Tris hydrochloride (pH 7.4) and then centrifuged at 23,000 rpm in a Beckman SW27 rotor for 18 h at 4°C. The virus pellet was suspended in 0.15 M NaCl–0.02 M Tris hydrochloride (pH 7.4) and subjected to isopycnic centrifugation on a linear 15 to 55% (wt/vol) sucrose gradient over a 65% sucrose cushion in the same buffer at 23,000 rpm in a Beckman SW40 rotor for 18 h at 4°C. Fractions (21 to 22) were collected from a side puncture near the bottom of the tube, and refractive indices were determined. The position of virus within the gradient was determined either by the endogenous DNA polymerase reaction (23) or by Southern analysis of aliquots. For Southern analysis, samples of equal volume from the gradient fractions were incubated in a sodium dodecyl sulfate–pronase solution, extracted with 1:1 phenol–chloroform, and precipitated with ethanol. Samples were then subjected to agarose gel electrophoresis and transferred to nitrocellulose, and viral DNA was detected with radioactively labeled probes as described for intracellular viral DNA.

RESULTS

Transfected LMH cultures support higher levels of DHBV DNA synthesis than do HuH-7, Hep G2, or IQ1a cultures. Previous reports of DHBV production by transiently transfected HuH-7 (28) and Hep G2 cultures have indicated that only low levels of infectious virus are released. Since both of these cell lines are of human liver origin, it was thought that an avian cell line might provide better yields of the avian virus. Therefore, to compare levels of intracellular viral DNA synthesis intermediates and virus release, we transfected cultures of HuH-7, Hep G2, IQ1a, and LMH cells with a plasmid containing a tandem dimer of DHBV DNA (pSP.DHBV5.1Galx2). Viral DNA extracted from cytoplasmic core particles isolated from transfected cells on day 5 posttransfection is shown in Fig. 2. (Viral DNA synthesis in all these cells peaked at 4 to 5 days posttransfection [data not shown].) Remarkably, both RC and double-stranded linear

DNA forms produced in transfected LMH cells were visible in ethidium bromide-stained agarose gels (Fig. 2A). By Southern analysis (Fig. 2B and C), it was clear that the synthesis of the three major forms of intracellular DNA intermediates (RC, double-stranded linear, and single-stranded linear) was markedly enhanced in the LMH cells compared with the HuH-7, Hep G2, or IQ1a cells. A 5- to 10-fold-greater amount of replicative DHBV DNA was seen in LMH cultures than in any of the other cultured cell lines.

Control of DHBV pregenome synthesis by the CMV-IE promoter does not dramatically affect DHBV DNA synthesis in either LMH or HuH-7 cultures. We attempted to improve DHBV DNA synthesis within transfected cultures by replacing the cognate viral core promoter with the CMV-IE enhancer-promoter element to control transcription of the RNA pregenome (pCMVDHBV). The CMV-IE enhancer-promoter has been reported to be highly active in a variety of cell types (5, 8, 33, 35), and in addition, Seeger et al. (33, 35) have shown that Hep G2 cells, NIH 3T3 cells, and turkey fibroblasts transfected with a plasmid containing the CMV-IE promoter (to direct the transcription of a cloned WHV insert) all produce infectious virus. LMH cells transfected with either pCMVDHBV or pSP.DHBV5.1Galx2 contained more intracellular DHBV DNA intermediates than did HuH-7 cells transfected with the same DNAs. Only a very slight enhancement in the levels of these intermediates was observed, however, when pCMVDHBV was used rather than pSP.DHBV5.1Galx2, and this difference was observed in both LMH and HuH-7 cells (data not shown).

In the generation of pCMVDHBV from pSP.DHBV5.1Galx2, the coding capacity of the transfected DNA for the DHBV precore protein was eliminated. To determine whether this mutation could affect DNA replication in LMH cells, cultures were transfected with pSP.DHBV5.1Galx2, pCMVDHBV, or an *Eco*RI dimer construct similar to pSP.DHBV5.1Galx2 but containing the precore mutation described by Chang et al. (6). Essentially no difference in DHBV DNA synthesis was observed for the two dimer-

FIG. 3. Production of infectious DHBV particles by transiently transfected HuH-7 and LMH cultures. Medium harvested from HuH-7 and LMH cultures at day 5 following transfection with pCMVDHBV was analyzed for the presence of both physical virus particles (A) and infectious virus (B). In panel A, virus particles present in the culture medium were sequentially sedimented through a sucrose step gradient and separated from free core particles by isopycnic centrifugation on a linear sucrose gradient as described in the text. Fractions were collected through a single puncture near the bottom of the centrifuge tube, and DNA was extracted from aliquots of each fraction for Southern blot analysis. Shown here are the peak fractions in which virus particles were found in the gradients. M, ca. 50 pg each of a double- and single-stranded 3-kilobase DHBV fragment. In panel B, duplicate primary cultures of duck hepatocytes were infected by incubation in medium containing 0.5 ml of clarified culture fluids from the transfected cultures, or 5 or 50 μ l of DHBV-positive serum. Suramin was added to all hepatocyte cultures, and PFA was added to selected hepatocyte cultures following a 24-h viral adsorption period. The appropriate drugs were maintained in the medium until the cells were harvested, and total cellular nucleic acids were extracted at day 3 or 8 postinfection for Southern blot analysis with a DHBV-specific DNA probe. Symbols: -, no PFA present in culture medium; +, 1 mM PFA present in culture medium. RC, DSL, and SS are defined in the legend to Fig. 2.

containing plasmids (data not shown). Thus, the lack of a drastic increase in DHBV DNA synthesis when using the CMV-IE promoter to drive transcription was not due to a counterbalancing inhibitory effect associated with the absence of the precore polypeptide. (It should be noted that reverse transcription of the pregenome following pCMVDHBV transfection could, in theory, restore the pregenome coding capacity by generating viral CCC DNA via the intracellular CCC DNA amplification pathway [45, 47; J. Summers, personal communication]. Because of the large amounts of plasmid DNA in transfected cultures, it is uncertain whether this pathway is operative in our experiments.)

Transfected LMH cultures release greater quantities of infectious DHBV than do HuH-7 or Hep G2 cultures. To quantitate the levels of virion production by transfected cells, the particles were subjected to isopycnic sucrose density gradient centrifugation. DHBV normally bands at ca. 1.16 g/ml, whereas viral cores, commonly found in transfected culture fluids, sediment through the gradients used in these experiments. Analysis revealed that LMH cells transfected with either pCMVDHBV or pSP.DHBV-5.1Galx2 consistently released approximately 15-fold more virus than do similarly transfected HuH-7 cells (Fig. 3A). Transfected Hep G2 and IQ1a cultures produced virus particles at levels comparable to or less than those produced by HuH-7 cultures (data not shown). By comparison with the hybridization signal produced by a cloned viral DNA marker, we estimated that 125 to 150 pg of RC DNA was present in virions found in 1 ml of the transfected LMH culture medium (i.e., ca. 5×10^7 virus particles) and that only about 10 pg of RC DNA was present in virions from 1 ml of transfected HuH-7 medium. A pool of DHBV-positive

duck serum used in the infectivity studies (see below) contained 7 to 8 ng of RC DNA per ml that was present in virus particles.

The infectivity of the virus produced by the cell lines following transfection with pCMVDHBV was tested, using clarified medium, in primary cultures of duck hepatocytes (Fig. 3B). Suramin was added to all duck hepatocyte cultures after a 24-h virus adsorption period to prevent subsequent secondary rounds of infection (26). Selected cultures were also incubated in PFA, an inhibitor of intracellular viral DNA synthesis (22, 37), to show that replicative forms were produced de novo. Cultures were harvested at days 3 and 8 postinfection. Although RC viral DNA was present in cultures on day 3, this DNA must have been derived largely from the original virus inoculum because this DNA was much reduced or completely absent by day 8 in cultures incubated in PFA. In the absence of PFA, the amount of viral DNA increased with time and, in addition to 3-kilobase-pair (kbp) RC, 3-kbp double-stranded linear, and 3-kilobase single-stranded DNA, supercoiled (CCC) DNA was plainly visible by day 8 postinfection. Therefore, the virus produced by the transfected cultures was infectious. Furthermore, we estimated that 1 ml of medium harvested from transfected LMH cultures contained ca. 10 times the amount of infectious virus as did HuH-7 culture medium, equivalent to ca. 30 to 40 μ l of the DHBV-positive serum used as a control.

The observations that the transfected LMH cells produced infectious DHBV particles and that these cells contained very high levels of intracellular viral DNA replicative intermediates led us to examine the possibility that the LMH cells could be infected with DHBV. LMH cultures incubated in either clarified medium from transfected LMH cells known to produce infectious DHBV or pooled DHBV-positive sera

FIG. 4. Complementation and recombination between cotransfected DHBV-containing plasmids in LMH and HuH-7 cultures. Cultures of LMH and HuH-7 cells were cotransfected with a total of 10 μ g of plasmid DNA in the following combinations: 5 μ g of pSP.DHBV5.1Galx2 plus 5 μ g of pTTW-1 (see text); 5 μ g of pSP.DHBV5.1RV718 plus 5 μ g of pTTW-1; 5 μ g of pSP.DHBV5.1RV718 plus 5 μ g of pCMVpol; and 5 μ g of pSP.DHBV5.1RV718 plus 5 μ g of pCMVΔPpol. On day 5 posttransfection, cytoplasmic core DNA was extracted from the cells for Southern blot analysis on the native DHBV DNA (A) to assay complementation between cotransfected mutant DHBV DNA molecules. The extracted DNA was digested with *EcoRV* with cleavage monitored by the inclusion of λ DNA in each reaction (B). In panel B, exposures of the autoradiograms varied: 5.1 x 2, 23 h at room temperature; RV718 + pol and RV718 + ΔPpol, 96 h at -80°C with a Du Pont Cronex Lightning-Plus intensifying screen. RC, DSL, and SS are defined in the legend to Fig. 2. 5.1x2, pSP.DHBV5.1Galx2; RV718, pSP.DHBV5.1RV718; pol, pCMVpol; ΔPpol, pCMVΔPpol. M (panel A), 50 pg each of double- and single-stranded 3-kilobase DHBV fragment; M (panel B) *HindIII*-digested bacteriophage λ DNA size markers.

failed to produce any DHBV viral DNA by day 9 postadsorption (data not shown). Thus, the LMH cell line is apparently not susceptible to DHBV infection.

Complementation efficiency for DHBV mutant genomes is greater in LMH than in HuH-7 cultures. Because the transiently transfected LMH cultures synthesized much higher levels of DHBV DNA than did similarly transfected HuH-7, Hep G2, or IQ1a cultures, we asked whether complementation efficiency between cotransfected mutant DHBV genomes might also be high in this avian cell line. Construction of plasmids used in these assays is described in Materials and Methods and shown in Fig. 1. pSP.DHBV5.1RV718 contains a frameshift mutation to eliminate polymerase activity. pCMVpol contains DHBV DNA of less than unit length, encoding an intact polymerase open reading frame with transcription under the control of the CMV-IE promoter; S and pre-S proteins but not viral core proteins should also be encoded in this region of DHBV DNA. As expected, LMH or HuH-7 cells transfected with pSP.DHBV5.1RV718 or pCMVpol, alone, did not synthesize viral DNA (see Fig. 4A and 6A). However, cotransfection of the polymerase-negative construct, pSP.DHBV5.1RV718, together with pCMVpol resulted in DHBV DNA synthesis. DHBV DNA levels in the cotransfected LMH cultures were consistently closer to the levels reached following single transfection with wild-type (pSP.DHBV5.1Galx2) DNA than was the case when a similar comparison in HuH-7 cells was made (e.g., ca.10% in LMH cells versus <1% in HuH-7 cells in the experiment shown in Fig. 4A). Thus, a higher level of apparent complementation for DHBV polymerase occurred in the LMH cultures than in the HuH-7 cultures.

To establish that virus released from the cotransfected culture was, in fact, of the mutant genotype, clarified medium from cultures transfected with pSP.DHBV5.1Galx2 or cotransfected with pSP.DHBV5.1RV718 and pCMVpol was used to infect cultures of primary duck hepatocytes. Suramin was added at 24 h postinfection to prevent further virus uptake. Both total and CCC DNAs were extracted from the hepatocytes at days 3 and 8 postinfection. Although input virus adsorbed to the hepatocytes, and CCC DNA was generated in both cases, viral replication (as indicated by an increase in DNA levels from day 3 to day 8) was detected only when wild-type virus harvested from cultures transfected with pSP.DHBV5.1Galx2 was the inoculum; therefore, the cotransfected cultures did not produce detectable amounts of wild-type virus (data not shown). The amount of CCC DNA was approximately 20-fold greater at day 3 postinfection with the wild-type virus (Fig. 5), a difference that may be attributed to a lower inoculum of mutant viruses as well as to early CCC DNA amplification in cultures infected with the wild-type virus (45). These results suggest that efficient infection by mutant viral genomes did occur and that the CCC DNA template retained the original *pol* mutation and could not support viral DNA replication; they also confirm that CCC DNA amplification, which occurred in wild-type- but not *pol* mutant-infected hepatocytes, requires *pol* gene expression (Fig. 5).

Homologous recombination between DHBV DNA molecules occurs in both transfected LMH and HuH-7 cells. Although, in our experiments, replication-competent (wild-type) virus was not detected in the media of cultures cotransfected with mutant genomes (Fig. 5), and previous studies (18, 31) have

FIG. 5. Infectivity assay of virus released from LMH cultures transiently transfected with pSP.DHBV5.1Galx2 or pSP.DHBV5.1RV718 plus pCMVpol DNA. Primary cultures of duck hepatocytes were infected with 1 ml of clarified medium from transfected cultures. Suramin was added following a 24-h adsorption period, and CCC DNA was extracted at days 3 and 8 postinfection for analysis on Southern blots by hybridization with a DHBV-specific genomic DNA probe. The exposure times of the autoradiograms were 23 h (left) and 8 days (right). RC, DSL, and SS are defined in the legend to Fig. 2. RV718, pol, 5.1 $\times$ 2 are defined in the legend to Fig. 4.

failed to detect homologous recombination between DHBV genomes in HuH-7 cultures, we assayed for DHBV recombination in both LMH and HuH-7 cultures. In these cultures, cotransfection of pCMV Δ Ppol, a promoterless polymerase construct (Fig. 1E), with pSP.DHBV5.1RV718, resulted in DHBV DNA production, but at much lower levels than observed following cotransfection of pCMVpol with pSP.DHBV5.1RV718 (Fig. 4A). To determine whether this newly synthesized DHBV DNA may have been produced, to some extent, as a result of homologous recombination of input viral DNAs to regenerate a wild-type genotype capable of expressing the DHBV polymerase, we assayed for the presence of a particular *EcoRV* restriction enzyme site in the RC DNA harvested from transfected LMH cultures which was not present in pSP.DHBV5.1RV718 (Fig. 4B). RC DHBV DNA extracted from core particles in cultures transfected with pSP.DHBV5.1Galx2 (wild-type) contains two *EcoRV* sites, at nucleotides 718 and 2652. Therefore, digestion of RC DNA with *EcoRV* resulted in fragments of 1.9 and 1.1 kbp. In the mutant, pSP.DHBV5.1RV718, the *EcoRV* site at nucleotide 718 was eliminated, and, thus, digestion of RC DNA with *EcoRV* should generate only a 3.0-kbp fragment if a mutant DHBV genotype was maintained. Clearly, both 1.9- and 1.1-kbp fragments were present following *EcoRV* digestion of DNA from the cotransfected LMH cultures, and the ratio of the 1.9- and 1.1-kbp fragments to the 3.0-kbp fragment differed for the two distinct cotransfections. (DHBV DNA harvested from HuH-7 cultures was not examined by this method because of the extremely low levels of DHBV DNA following cotransfection with any of the polymerase constructs.) Although this result indicates that

FIG. 6. Complementation and recombination between DHBV DNA sequences within an LMH subline (*pol*-7) containing a single functional integrated copy of the DHBV polymerase gene. Duplicate cultures of *pol*-7 cells were transfected with pSP.DHBV5.1Galx2, pSP.DHBV5.1RV718, or pCMVpol, and cytoplasmic core DNA was extracted on day 5 posttransfection for Southern blot analysis by hybridization with a DHBV-specific genomic DNA probe (A). The extracted DNA from cultures transfected with either pSP.DHBV5.1Galx2 or pSP.DHBV5.1RV718 was digested with the restricted enzyme *EcoRV* (B). The exposure times on the autoradiograms in panel B were 4 h for 5.1x2 and 48 h for RV718. RC, DSL, and SS are defined in the legend to Fig. 2. 5.1x2, RV718, and pol are defined in the legend to Fig. 4. M (panel A), ca. 50 pg of double-stranded 3-kbp DHBV fragment. M (panel B), *Hind*III-digested bacteriophage λ DNA size markers.

recombination may be an explanation for the "complementation" observed in cotransfections with the promoterless plasmid, it should be noted, first, that a contribution of *pol* gene expression by cryptic promoters in pCMV Δ Ppol was not ruled out and, second, that wild-type recombinants were too infrequent to be detected in assays of infectious virus production.

In the transient-transfection assays, it was not possible to determine how many copies of a given DHBV DNA molecule were taken into any single cell. Therefore, in the complementation experiments involving cotransfection of two distinct DHBV DNAs, we could not control the actual amount of DHBV DNA in any given cell, but we believed that if the copy number of at least one input plasmid DHBV DNA within each transfected cell was reduced, a reduction in homologous recombination might occur. To do this, an LMH culture was transfected with the *pol* construct, pCMVPN1-2, and neomycin-resistant clones were selected. In one of the transformed clones, DHBV DNA synthesis occurred following single transfection either with wild-type (pSP.DHBV5.1Galx2) plasmid or with the polymerase-negative construct, pSP.DHBV5.1RV718, but not following transfection with pCMVpol (Fig. 6A). From the *EcoRV* restriction endonuclease analysis (Fig. 6B) and the lack of wild-type virus production by these cells when transfected with the polymerase pSP.DHBV5.1RV718 (data not shown), DHBV DNA recombination was below detectable levels in the transfected *pol*-7 cell line. By examination of restriction enzyme digestion patterns on total cellular DNA extracted

from *pol-7* cells, we believe that only one copy of the *pol* gene is integrated into the host genome (data not shown).

DISCUSSION

The chicken LMH cell line was superior to the previously described (12, 13, 28) human HuH-7 and Hep G2 cell lines and to the quail IQ1a cell line for DHBV DNA synthesis following transfection with viral constructs. Higher levels of DHBV DNA were observed in (Fig. 2) and greater quantities of infectious DHBV were produced by (Fig. 3) transfected LMH cultures than were seen with any of the other liver cultures examined, whether or not the viral core promoter or the CMV-IE promoter was used to drive transcription of the pregenomic RNA. It was not clear at what level(s) cellular regulation of DHBV DNA synthesis occurs. Differences in the cell lines may exist, for instance, in template RNA synthesis or in the packaging of the RNA into the core particles in which viral DNA synthesis occurs. Evidence for posttranscriptional effects as well as virus-specific differences in the capacity for virus DNA synthesis was implied by the observation that transfected HuH-7 cells supported much higher levels of WHV DNA synthesis than did LMH cells (data not shown). This difference was unlikely to be at the level of pregenome transcription, since the CMV-IE promoter was used for both WHV and DHBV.

Complementation efficiency between a polymerase-negative DHBV mutant (pSP.DHBV5.1RV718) and a polymerase-expressing construct (pCMVpol) was also higher in the cotransfected LMH cells than in the HuH-7 cells, as indicated by DHBV DNA levels in the transfected cultures (Fig. 4A). Surprisingly, cotransfection of pSP.DHBV5.1RV718 with a presumably promoterless polymerase construct (pCMVΔPpol) resulted in low levels of DHBV DNA replicative intermediates in both LMH and HuH-7 cultures. This may have occurred through a variety of mechanisms including, but not limited to, mutation of input DNA and homologous recombination. Direct reversion of the mutant DNA to a wild-type genotype or the introduction of a second-site compensatory mutation to give a wild-type phenotype have been described for an *in vivo* transfection system (19) and could be responsible for DHBV DNA synthesis. However, we do not believe this to be a valid possibility because we have never detected DHBV DNA replicative intermediates following single transfection with the mutant pSP.DHBV5.1RV718 (Fig. 4A). Therefore, the presence of both the promoterless polymerase-expressing plasmid pCMVΔPpol and the polymerase-negative construct pSPDHBV5.1RV718 were required for DHBV DNA synthesis to occur. Although homologous recombination between input DHBV genomes was not detected in transfected human liver cell lines (18, 31), the phenomenon has been detected for other transfected DNAs in many cell types (9, 29, 39, 42) and described after *in vivo* transfection of ducks with cloned DHBV regions (41). From the *EcoRV* digestion data presented in Fig. 4B, it is clear that homologous recombination between input plasmids containing DHBV sequences occurs at a very low level for all cotransfection experiments in the LMH cell line. With the generation of the LMH subline (*pol-7*), which is stably transformed with a single copy of the DHBV polymerase gene, we have been able to effectively eliminate any potential problem of homologous recombination.

Others have been unable to accurately determine recombination efficiency following *in vivo* transfection of ducks (41) because of the rapid spread of infectious virus produced

through recombination. This same limitation probably does not apply when using the cultured cell lines, as these cells are resistant to virus infection. Therefore, we estimated that in the LMH cultures the frequency of homologous recombination to generate a functional polymerase gene without disruption of replication of the viral genome by the introduction of other mutations was slightly less than 0.1%. Calculation of homologous recombination frequency for the HuH-7 cultures was not as straightforward; because of the much lower levels of viral DNA synthesis in these transfected cultures, analysis of the DNA from the co-transfected cultures by restriction mapping was technically difficult. In addition, we cannot rule out the existence of a cryptic promoter in the pCMVΔPpol construct which may be responsible for the synthesis of a low level of DHBV polymerase in any of the transfected cultures. Thus, the RC DNA seen in HuH-7 cultures may be due to either a minimal level of polymerase expression from pCMVΔPpol or homologous recombination.

Irrespective of these technical considerations, it is important that LMH cells produce amounts of virus that allow easy detection of replicative intermediates following addition of even small amounts of transfected cell culture fluids to primary duck hepatocyte cultures (Fig. 3B). This should facilitate genetic approaches to determining the role of viral gene products in the initiation and maintenance of chronic hepatocellular infections, as demonstrated by our preliminary analysis of the role of the virus-encoded reverse transcriptase in CCC DNA amplification.

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