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Synthesis of Hepadnavirus Particles That Contain Replication-Defective Duck Hepatitis B Virus Genomes in Cultured HuH7 Cells

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To evaluate the possibility of producing transducible replication-defective hepadnaviruses, cloned mutant duck hepatitis B virus genomes were tested both for virus antigen production and viral DNA synthesis following transfection into the human hepatoma cell line HuH7. Deletion of a *cis*-acting 12-nucleotide sequence implicated in viral DNA synthesis, direct repeat 1 (DR1), resulted in the loss of ability to synthesize both mature viral DNA and infectious virus. The ΔDR1 mutant, however, produced envelope and core antigens and was shown to provide *trans*-acting functions required for the assembly of infection-competent particles. Thus, mutants with mutations in viral genes could be rescued as DNA-containing viral particles after cotransfection with ΔDR1. The efficiency of rescue was influenced by the site of mutation. A mutant DNA encoding truncated core and envelope proteins not only was poorly rescued but also was able to suppress the production from a wild-type DNA of infectious virus.

The course of a number of diseases involving the mammalian liver, particularly inherited single-gene defects and viral diseases, might be significantly altered by genetic transduction of this organ. An approach under current investigation involves the use of recombinant retroviruses to transduce primary hepatocytes in culture, after which the cells are implanted into the peritoneal cavity (7, 22, 23). Another approach is to use a hepatotropic virus that can directly infect hepatocytes in vivo. Hepatitis B viruses (HBV) fit such a description (for a review, see reference 5), and these DNA viruses exhibit features that make them attractive as potential hepatic transducing vectors. They can be delivered to the liver via the circulation and are known to be expressed specifically in the liver. Hepadnavirus DNA persists in infected hepatocytes in a free closed-circular form in the nucleus (20), and recombination with chromosomal DNA appears to be rare.

Recent studies have demonstrated the biological activity of cloned genomic DNA from human HBV (2, 17, 19, 24) and the structurally and functionally homologous duck hepatitis B virus (DHBV) (4, 15). When plasmids containing tandem copies of these genomes are transfected into the cultured human hepatoma cell lines HepG2 and HuH7, respectively, infectious wild-type virus is produced. The transfecting DNA serves as a template for RNA production, and progeny viral DNA is produced via the reverse transcription pathway normally used in hepatocytes (18). In this communication we report the further use of the cloned DHBV genome and HuH7 cells in the development of a system for producing replication-defective HBV particles. We also report that a defective DHBV genome encoding truncated viral proteins can block the production in HuH7 cells of wild-type virus.

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MATERIALS AND METHODS

Plasmids. Modifications of the wild-type DHBV sequence were produced in plasmids containing a single copy of the genome. The ΔDR1 genome was produced by first inserting the distal portion of the wild-type genome, contained between *Bam*HI (nucleotide 1658) and *Eco*RI (nucleotide 3021), into an Acc$^-$ pBR322 derivative (produced by *Acc*I digestion, fill-in, and ligation, thus deleting the pBR322 sequence between nucleotides 651 and 2246). Next, a portion of the wild-type DHBV genome including DR1 (nucleotides 2535 to 2546) was excised from the recombinant plasmid by using *Afl*II and *Acc*I (cleaving at nucleotides 2526 to 2577, respectively) and was replaced by a double-stranded synthetic DNA segment precisely deleted of the 12 base pairs making up DR1. The desired plasmid was readily identified by the presence of a new *Hin*fI site at the position of the DR1 deletion. Finally, the proximal portion of the DHBV genome (nucleotides 0 to 1658), including adjoining plasmid sequences at the upstream end, was inserted as a *Bam*HI fragment into the unique *Bam*HI site. The Kpn$^-$ genome was produced by digestion of the wild-type genome with *Kpn*I, treatment with DNA polymerase I (large fragment) in the presence of all four deoxyribonucleoside triphosphates to remove the 4-base 3′ overhang, and closure of the plasmid with T4 DNA ligase. The Kpn$^-$ Sph$^-$ genome was produced from a dimer of the Kpn$^-$ genome by partial digestion with *Sph*I, DNA polymerase I (large fragment) treatment to remove the 4-base 3′ overhang, and ligation. Deletion at the appropriate *Sph*I site was determined by restriction analysis of several resultant clones. EcoRV$^-$ genomes (deleted of 2 base pairs) were produced by partial digestion of the wild-type genome with *Eco*RV, followed by incubation in a DNA polymerase I (large fragment) reaction mixture in the presence only of dGTP. Specific mutations were confirmed by sequencing. Recombinants containing insertions of bacteriophage λ DNA were generated by digestion of the wild-type genome with *Kpn*I, treatment of the linearized plasmid with DNA polymerase I (large fragment) in the presence of deoxynucleoside triphosphates, and ligation of either 180- or 1,000-base-pair *Hae*III fragments of bacteriophage λ.

All of the modified DHBV genomes were dimerized by using one of two strategies. The first involved the use of partial *Eco*RI digestion to linearize the plasmid at one of the two *Eco*RI sites flanking the genome. Linear sequences were identified in agarose gels and recovered by electroelution; they were then treated with bacterial alkaline phosphate and joined with the corresponding *Eco*RI-cut, gel-purified monomer genome. The second strategy of dimerization involved the joining of *Eco*RI-cut, gel-purified monomer in threefold molar excess at 30 μg/ml with *Eco*RI-bacterial alkaline phosphate-treated pSP65. In all cases, dimer clones were identified by restriction analysis of plasmid DNAs.

Cells, transfection, and immunofluorescence analysis. The cultured human hepatoma cell line HuH7 (14) was kindly supplied by K. Koike and was propagated in a medium composed of Hamm's medium F12 and Dulbecco modified Eagle medium (1:1, vol/vol) plus 10% fetal bovine serum (GIBCO Laboratories). HuH7 cells were transfected by the calcium phosphate procedure (6). Portions (3 μg) of each plasmid were applied to nearly confluent cells in 60-mm dishes. Indirect immunofluorescence analysis was carried out on cells that were fixed 5 days after transfection, at −20°C in 95% ethanol–5% glacial acetic acid. Fixed cells were incubated with rabbit antisera (kindly supplied by W. Mason) directed against either DHBV core protein or DHBV surface antigen. After being washed, the cells were incubated with rhodamine-conjugated goat anti-rabbit immunoglobulin G (Cappel Laboratories).

Isolation of viral core particles from transfected cells. Five days after transfection, cells were washed with phosphate-buffered saline and then lysed in 1 ml of a solution containing 10 mM Tris (pH 7.9), 1 mM EDTA, 0.1% Nonidet P-40, 50 mM NaCl, and 8% (wt/vol) sucrose. After incubation for 10 min at 37°C, the lysate was removed and centrifuged at 7,000 rpm for 10 minutes in a Sorvall HS-4 rotor to pellet nuclei and other insoluble material. Viral cores in the supernatant were then precipitated by addition of an equal volume of 16% polyethylene glycol 8000–1 M NaCl followed by incubation at 4°C for 30 min. The precipitate was collected by centrifugation at 4°C in an HS-4 rotor at 7,000 rpm. It was dissolved in 1 ml of buffer containing 50 mM Tris (pH 7.8), 100 mM NaCl, and 0.5 mM EDTA. The 10 μl of 1 M magnesium acetate was added, followed by 100 μg of DNase I (type IV; Sigma Chemical Co.). Following a 30-min incubation at 37°C, the reaction mixture was adjusted to contain, in order, 15 mM EDTA, 0.5% sodium dodecyl sulfate, and 500 μg of pronase per ml. After further incubation for 30 min at 37°C, the sample was extracted with an equal volume of phenol, and nucleic acids in the aqueous phase were precipitated with ethanol. The precipitated material was redissolved in sample buffer, and, typically, one-third of the sample was applied to the slot of an agarose gel.

Primary hepatocytes. Cultures of primary duck hepatocytes were prepared by perfusion with collagenase as previously described (21). To augment viral replication, the medium (Lebowitz medium no. 15) was supplemented with 1.5% dimethyl sulfoxide and serum was omitted (J. Pugh and J. Summers, unpublished observations). Under these conditions, multiple rounds of infection occur, allowing for amplification of the infecting virus. Cells were infected within 3 days postplating by exposure to virus-containing culture medium for 24 h, followed by a medium change. Cells were assayed for viral replication after 8 or 10 days, as previously described (21).

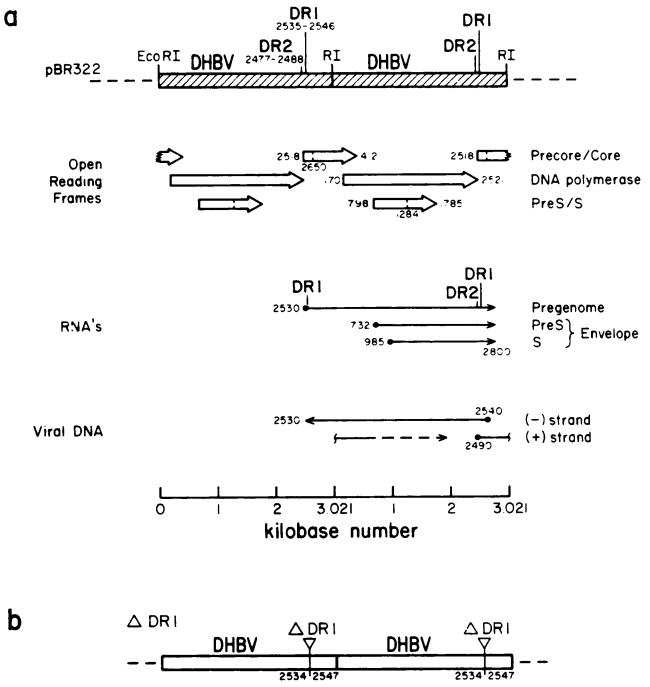

FIG. 1. Genetic and functional map of a dimer of DHBV DNA. (a) Location of copies of DR1 and DR2 in the dimerized *Eco*RI linear DNA inserted into pSP65. The positions of the open reading frames are shown, beginning with the first ATG in each frame (10). Vertical dashed lines correspond to internal ATGs that separate the open reading frames into the precore and core, or pre-S and S domains. The positions of the 5′ ends of RNA transcripts and their probable functions according to Buscher et al. (1) are shown below, as well as the positions of the 3′ and 5′ ends of the minus and plus strands. (b) Configuration of the dimer DNA containing the deletion of DR1 (ΔDR1).

RESULTS

The strategy for synthesizing replication-defective DHBVs involved the construction of two different types of mutant genome. The first is a packaging genome; this mutant genome should be capable of providing in *trans* all viral functions necessary for packaging pregenomic RNA into virus particles and for reverse transcription of the RNA into viral DNA; pregenomic RNA transcribed from this mutant genome, however, should itself fail to direct the production of a functional DNA copy, enabling the production by complementation of a helper-free stock of virus particles containing a second, defective DNA genome. The second type consists of defective genomes; these mutant genomes should not by themselves be capable of replication, but should be able to express a pregenomic RNA containing the *cis*-acting sequences required both for inclusion of the RNA into virus particles and for reverse transcription into DNA. Included within the defective class of mutants are recombinant genomes in which portions of the viral genome encoding functions that can be supplied in *trans* are replaced with a heterologous sequence.

For construction of a packaging genome, we took advantage of previous observations that two 12-nucleotide direct repeats in the DHBV genome, called DR1 and DR2, play a critical role in reverse transcription of pregenomic RNA into the circular, partially double-stranded DNA genome (Fig. 1a). In particular, DR1 is the site in pregenomic RNA at which initiation of minus-strand (first-strand) DNA synthesis

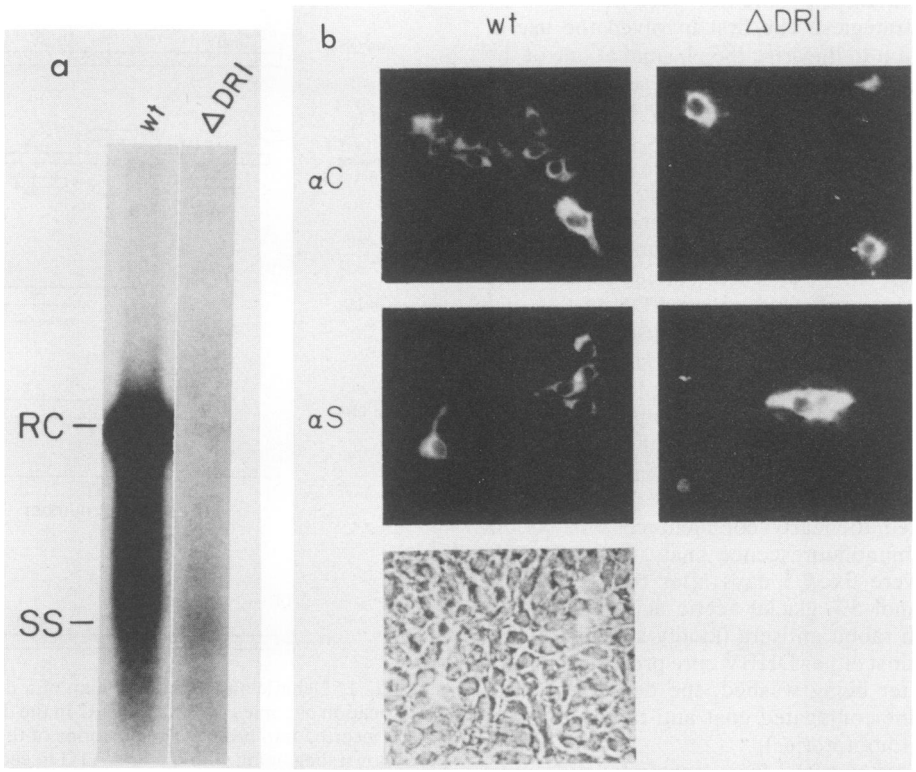

FIG. 2. (a) Transfection of HuH7 cells with plasmids containing wild-type or ΔDR1 dimers. Plasmid DNA (3 μg per dish) containing wild-type (wt) or ΔDR1 dimer was transfected into 2×10^6 HuH7 cells on 60-mm plastic dishes and incubated at 37°C. After 5 days, cell layers were washed with 2 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.45)–0.15 M NaCl (HBS) and viral DNA replicative intermediates were extracted as described in Materials and Methods. Viral DNA from one-third of a dish was electrophoresed through a 1% agarose gel, denatured, and blotted onto a nylon filter (Amersham Corp.). The filter was hybridized with [^{32}P]RNA prepared by transcription of a pSP65 plasmid containing the DHBV genome cloned at the *Eco*RI site in an orientation to yield plus-strand RNA transcripts (20). After hybridization and washing, the filter was exposed to Kodak X-AR films at −70°C with an intensifying screen. The migration positions of single-stranded minus strands (SS) and relaxed-circular (RC) forms are indicated. (b) Five days after transfection as in panel (a), cells were fixed and assayed for viral core antigen (αC) or surface antigen (αS) by immunofluorescence. All exposures were for identical times. A phase-contrast photograph of the HuH7 cell layer is shown for comparison.

occurs (13), whereas DR2 is the site in minus-strand DNA at which initiation of plus-strand (second-strand) DNA synthesis occurs (9). DR1 also functions to provide the primer for second-strand synthesis: this primer is a short RNA which is derived from the 5′ terminus of the genome and which is complementary to DR2 at its 3′-terminal DR1 sequence (8). Since DR1 is thus involved with the synthesis of both minus and plus DNA strands, we elected to precisely delete this sequence. Because every base in the DHBV genome is used to direct the synthesis of a protein, this alteration necessarily affects a viral product: it removes four residues from the NH_2-terminal portion of the precore protein (Fig. 1a). The normal function of this protein remains undefined, but previous studies indicate that it is dispensable to both viral replication and infectivity (16). Therefore, we hoped that the small deletion programmed within the precore would not affect the synthesis of the virus.

ΔDR1 dimer is defective in production of a packaged relaxed-circular genome. Two copies of the ΔDR1 genome were inserted in tandem into a pBR322 plasmid backbone (Fig. 1b) to permit transcription from the plasmid of the same terminally redundant pregenomic RNA that is normally produced from a single closed-circular viral genome. The ΔDR1 dimer plasmid and a wild-type dimer inserted into a pSP65 plasmid (15) were individually transfected into the human hepatoma cell line HuH7 by the calcium phosphate method, and viral core particles were isolated from the cells 5 days later. DNA was extracted from the particles, fractionated in an agarose gel, transferred to a membrane filter, and hybridized with an SP6-programmed genomic plus-strand RNA probe (20). When the plasmid containing the wild-type DHBV dimer was transfected, an intense hybridization signal was observed, extending from the migration position of single-stranded, minus-strand genomic DNA to a discrete band at the position of relaxed-circular genomic DNA (Fig. 2A) (11). This latter species is a form of the DNA genome found in infectious virus particles (12). This result agrees well with previous observations that following transfection with wild-type dimer, HuH7 cells produced infectious virus (15). In striking contrast, following transfection with ΔDR1 dimer, only a very faint hybridization signal was observed, at the migration position of minus-strand DNA. No signal was detectable at the migration position of relaxed-circular DNA. Thus, the transfected ΔDR1 genome failed to be converted into a packaged relaxed-circular DNA molecule.

Packaged relaxed-circular genomes are produced after cotransfection of defective dimer genomes with ΔDR1 dimer. Although ΔDR1 itself failed to direct the synthesis of a relaxed-circular DNA, we tested whether it could supply functions sufficient to permit the replication of other cotransfected, defective DHBV genomes. As a first examination, an

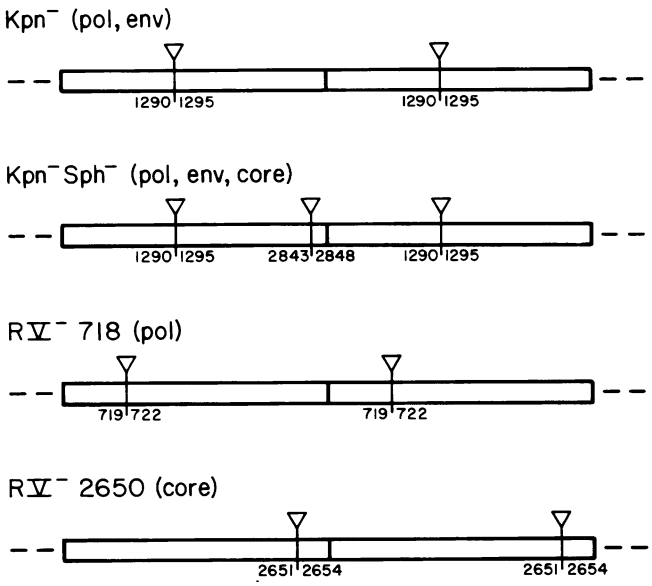

FIG. 3. Dimer mutants of DHBV defective in viral gene products. Symbols: □, *Eco*RI linear viral DNA; – – –, vector DNA. The numbers indicate the nucleotides joined as a result of a deletion. Shown in parentheses are the viral genes disrupted by the respective frameshift mutations.

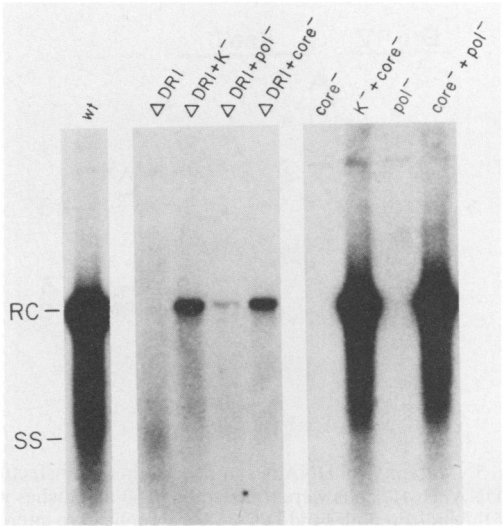

FIG. 4. Complementation of mutants for DNA synthesis. HuH7 cells were transfected in 60-mm plastic dishes with 3 mg each of the indicated plasmid DNAs. After 5 days, transfected cultures were harvested and viral DNA replicative intermediates were isolated and assayed as in Fig. 2a. The viral DNAs indicated are the wild type (wt), ΔDR1, Kpn⁻ (K⁻), RV-718 (pol⁻), and RV-2650 (core⁻).

indirect immunofluorescence study of transfected HuH7 cell populations was carried out with antisera directed against core protein and surface antigen. Both of these products were detected in the ΔDR1-transfected HuH7 cells, with an overall frequency and signal intensity similar to those observed following transfection of the wild-type dimer (Fig. 2b).

Next, a series of cotransfections were carried out, transferring simultaneously in coprecipitates plasmids containing the ΔDR1 dimer and dimers of the defective genomes shown in Fig. 3. These genomes contain small deletions engineered to produce a translational frameshift in one or more of the open reading frames encoding the major viral gene products. When *pol*⁻ *env*⁻, *pol*⁻, and core⁻ genomes were individually cotransfected with ΔDR1, relaxed-circular viral DNA genomes were detectable in virus cores isolated 5 days following transfection (Fig. 4). When core⁻ or *pol*⁻ genomes were transfected alone, no such product was observed, consistent with the prediction that these genomes would fail, respectively, to produce viral cores needed to package a genome or *pol* product(s) needed to copy pregenomic RNA into DNA. In the former case, the lack of production of stable nucleocapsids was verified by the absence of immunofluorescent staining of fixed core-transfected cells. Thus, ΔDR1 supplies core and *pol* functions which permit the packaging and reverse transcription of defective pregenomes produced by the cotransfected DNA. As further evidence that deficient functions of one genome can be supplied by cotransfection of a second complementary genome, two plasmids, each containing viral DNA defective in different viral genes, were cotransfected: a *pol*⁻ *env*⁻ genome or a *pol*⁻ genome was cotransfected with a core⁻ mutant. These genomes individually failed to produce relaxed-circular DNA, but following cotransfection a relaxed-circular DNA was readily observed to be produced within intact cores. The amount of product from these cotransfections is similar to that produced by transfection of wild-type dimer and appears to be at least an order of magnitude greater than that produced by the ΔDR1 cotransfections. The reason for this difference in efficiency of complementation remains unexplained.

The relaxed-circular genomes produced by cotransfer are defective genomes. To determine the genotype of the packaged genomes present as relaxed-circular DNA in ΔDR1 cotransfections, we carried out restriction analysis on the packaged viral DNA. Because the defective genomes are, in each case, deficient in a restriction site at the position of alteration (Fig. 3), the presence of a given genome in the DNA sample could be detected as an altered cleavage pattern by the appropriate enzyme. When DNA from wild-type dimer transfections was incubated with either *Eco*RV or *Kpn*I, fractionated in an agarose gel, and blotted, a pattern of cleavage products was observed that agrees with the known restriction sites for these enzymes in the DHBV genome, *Eco*RV cleaving at two positions (nucleotides 718 and 2650) and *Kpn*I cleaving once (nucleotide 1298) (Fig. 5). When DNA was similarly examined from cotransfections of ΔDR1 with either core⁻, or *pol*⁻ *env*⁻, different patterns were observed. In the case of core⁻, *Eco*RV digestion produced a major product corresponding in size to linear genomic DNA, indicating cleavage at only one site. In the case of *pol*⁻ *env*⁻ following *Kpn*I digestion, relaxed circles were the major product detected, indicating failure of cleavage with this enzyme. These results indicate that the relaxed-circular DNA in the viral core particles is derived primarily from the defective genomes in cotransfections with ΔDR1.

In restriction analysis of both cotransfections we also observed small amounts of cleaved products corresponding in size to wild-type fragments. We believe that these are the products of digestion of ΔDR1-containing plasmid DNA (wild-type pattern for *Eco*RV and *Kpn*I) present as a contaminant in the subviral core material. We consistently observed small amounts of such contaminating transfected DNA, despite exhaustive DNase I treatment of core preparations. Consistent with the identity of this DNA as a contaminant, it was observed regardless of whether the

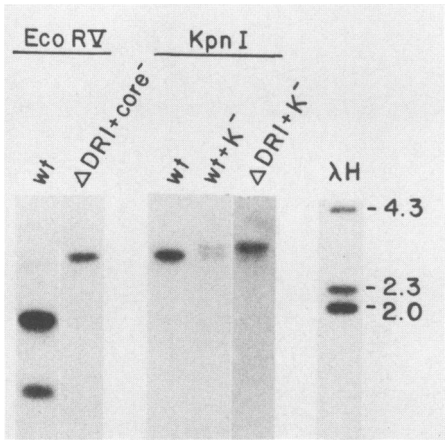

FIG. 5. Mutant viral DNA is synthesized in cotransfections with ΔDR1 DNA. HuH7 cells were transfected in 60-mm dishes with 3 μg each of the indicated plasmid DNAs. Viral replicative intermediates were isolated and digested with either *Eco*RV (left panel) or *Kpn*I (right panel), and the products were analyzed by electrophoresis through a 1% agarose gel, denatured, and transferred to a nylon membrane. The membrane was then hybridized to a [^{32}P]RNA probe prepared as described for Fig. 2a, except that the probe was of minus-strand polarity to detect the plus strand, which is found only in relaxed-circular DHBV DNA. A cotransfection of wild-type (wt) and Kpn$^-$ (K$^-$) plasmids was included to demonstrate the resolution of their *Kpn*I digestion products (linear and circular 3-kilobase-pair DNA) in this gel. Plasmid designations are as in the legend to Fig. 4.

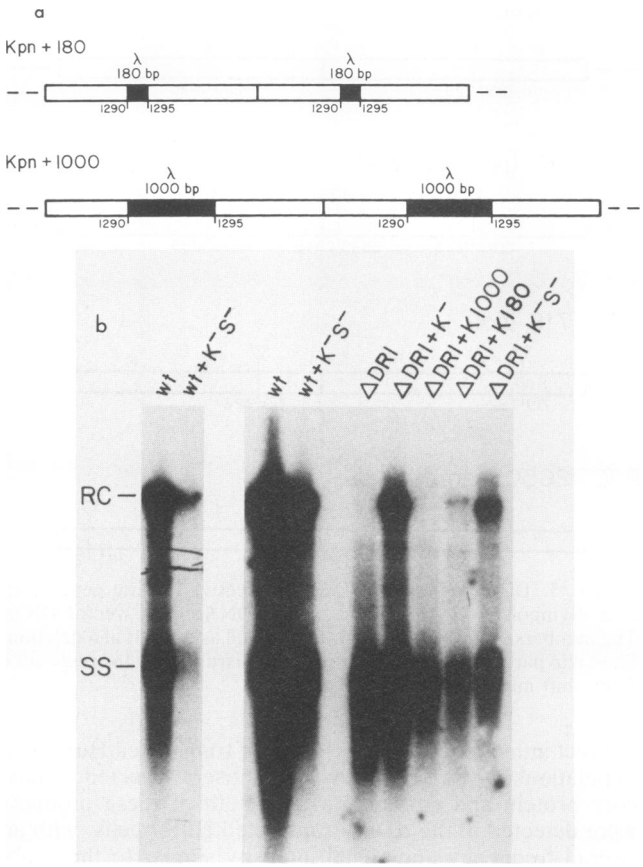

FIG. 6. (a) Structure of recombinant DHBV DNAs. Symbols: ■, inserts consisting of *Hae*III fragments of λ DNA; □, linear *Eco*RI dimer DHBV sequences. The numbers above the boxes indicate the approximate length of the insert in base pairs. The numbers below the boxes indicate the DHBV nucleotide joined to the insert. (b) Viral DNA synthesis in cotransfections. Viral DNA-containing plasmids (3 μg each per dish) were transfected into HuH7 cells and incubated at 37°C. After 5 days, viral DNA replicative forms were extracted and separated by electrophoresis through a 1.5% agarose gel. DNA was denatured, transferred to a nylon filter, and hybridized to a ^{32}P-labeled plus-strand viral RNA for detection of the minus strand by autoradiography. The exposure time of the left panel (same as the first two lanes of the right panel) was one-sixth that of the right panel. Viral DNAs used for transfection were wild-type (wt), ΔDR1, Kpn$^-$ (K$^-$), Kpn$^-$Sph$^-$ (K$^-$S$^-$), Kpn+180 (K180), and Kpn+1,000 (K1000). Notations are as in Fig. 2a.

transfected DNA could produce viral cores. The basis for the DNase resistance of this plasmid DNA is not known. It is conceivable, in cases when two plasmids have been cotransfected, that fragments are cleaved from wild-type genomes that may be formed at low frequency by a recombination event between two input genomes. Such an event must occur only at very low frequency, because we have not been able to detect wild-type virus infectivity in culture fluids of such cotransfections (data not shown).

A recombinant viral genome can be rescued following cotransfection with ΔDR1. Because defective DHBV genomes were rescued when cotransferred with ΔDR1, it seemed possible that recombinant genomes, containing non-viral sequences, could also be packaged and converted to relaxed-circular DNA. To examine this possibility, we constructed two recombinant genomes, each containing an *Hae*III fragment of bacteriophage λ DNA inserted into the DHBV genome at the unique *Kpn*I site at position 1290 (Fig. 6a). These two inserted fragments, 180 base pairs (K+180 genome) and 1,000 base pairs (K+1,000 genome), interrupt the continuity of both *pol* and *env* open reading frames.

Plasmids containing dimer forms of the two recombinant genomes were cotransfected with the ΔDR1 dimer plasmid into HuH7 cells, and viral cores were prepared after 5 days. When either ΔDR1 alone or ΔDR1 plus K+1,000 was transfected, no relaxed-circular DNA was detected (Fig. 6b). However, when ΔDR1 dimer was cotransfected with dimers of the genomes defective in *pol* and *env* (K$^-$) or in *pol*, *env*, and core (K$^-$S$^-$) (Fig. 3) or with the recombinant K+180 genome, relaxed-circular DNAs were detected. For K+180, the migration of the relaxed-circular DNA was retarded relative to that of the other genomes. This demonstrates that the larger-than-wild-type genome was packaged. The amount of this relaxed-circular species is substantially smaller than that of K$^-$ or K$^-$S$^-$, raising the possibility that there is a constraint upon packaging, perhaps owing to the size or location of the insertion. The failure to observe any packaged K+1,000 genome is consistent with such a hypothesis. In experimental support of such a constraint, we observed that the amount of pregenomic RNA, the substrate for packaging, was the same in cells transfected with dimers of wild-type, K+180, or K+1,000 (data not shown).

Assay of supernatants from transfected HuH7 cells for infectious virus. To assay for infectious virus produced by HuH7 cells transfected with DHBV genomes, we harvested supernatants from the cultures 5 days after transfection and incubated them overnight with freshly prepared primary cultures of duck hepatocytes (21). The hepatocytes were then assayed 10 days later for the presence of viral DNA by extraction of total DNA, agarose gel fractionation, and blot hybridization analysis. When supernatants from cells trans-

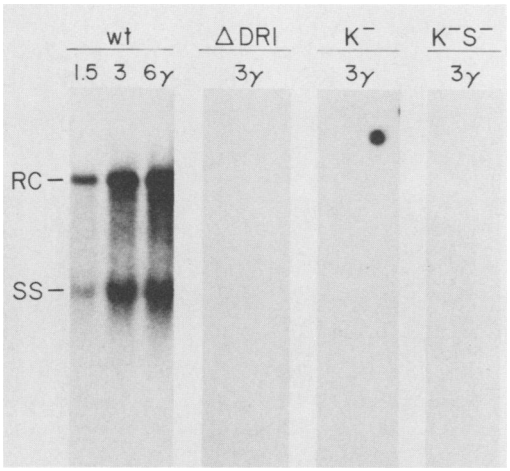

FIG. 7. Infectivity assays of HuH7 supernatants transfected with viral DNA-containing plasmids. HuH7 cells were transfected with the amounts of viral DNA indicated above the lanes and incubated at 37°C. Culture fluids removed from the cells between 3 and 5 days posttransfection (1 ml) were transferred onto fresh monolayers of primary duck hepatocytes in 3 ml of hepatocyte culture medium. After 24 h, the inoculum was removed and replaced with hepatocyte medium. Total DNA was extracted from each plate of hepatocytes after 10 days of additional incubation and assayed for viral DNA sequences by electrophoresis through a 1% agarose gel and blot hybridization. Designations of the DNAs used in the transfections are as in Fig. 6.

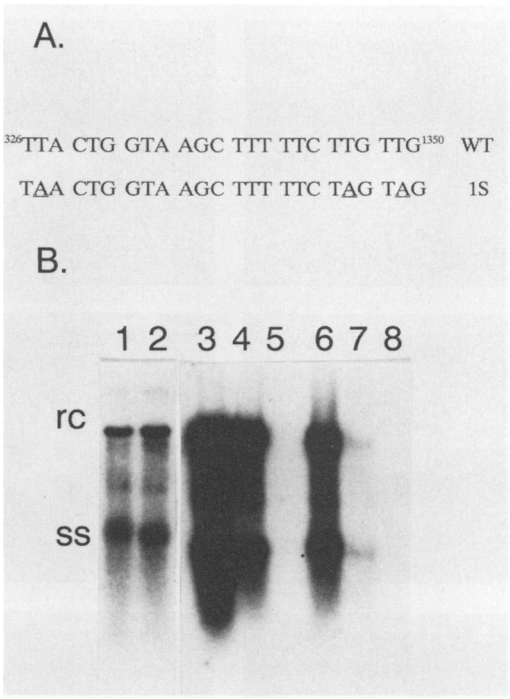

FIG. 8. Production of infection-competent particles carrying a mutant viral genome. (A) Single-base substitutions introduced into the envelope gene of DHBV. The sequence (WT) shown within the region coding for the S polypeptide was mutated at the three sites indicated by underlining to generate the mutant sequence (1S) containing termination codons within the S open reading frame without changing the amino acid sequence of the DNA polymerase. The mutant genome was inserted as a dimer into pSP65 and used in the transfection shown in panel B. (B) Southern blot hybridization of intracellular viral DNA replicative forms extracted from HuH7 cells transfected with mutant 1S (lane 1) or wild-type (lane 2) plasmids. Culture fluids collected between 3 and 5 days posttransfection (1 ml) from the HuH7 cells transfected with either wild-type or mutant 1S plasmids in the presence or absence of ΔDR1 plasmid DNA were used to infect primary hepatocytes. Protein-bound DNA (viral replicative forms) was extracted from the cells after 8 days and analyzed for viral DNA by blot hybridization. Cells were infected with 100 μl of virus-containing serum (lane 3) or with culture fluids from HuH7 cells transfected with the following plasmid DNAs: wild type (lane 4), mutant 1S (lane 5), wild type plus ΔDR1 (lane 6), mutant 1S plus ΔDR1 (lane 7), or no DNA (lane 8).

fected with wild-type dimer were tested, both minus-strand and relaxed-circular forms of the genome were observed in the hepatocytes (Fig. 7), demonstrating an ongoing process of viral replication in these cells. When supernatants from cells transfected with ΔDR1, K^-, and K^-S^- dimer were tested, no signal was detected in the hepatocytes (Fig. 7), indicating that infectious virus was not produced following transfection with these genomes. This is of particular significance for ΔDR1 because although its own pregenome appears to be packaged into cores and copied at low efficiency into a minus strand (Fig. 1a), no virus particles are produced that are infectious in hepatocytes. This is presumably due to the inability of this mutant to synthesize relaxed-circular DNA. Thus, this genome, which is able to provide viral gene products capable of rescuing defective genomes into packaged closed-circular form, is itself defective for production of infectious virus.

Production of infection-competent virions after cotransfection with ΔDR1 and an envelope mutant DNA. The release from HuH7 cells of infection-competent virus particles containing a mutant viral genome was demonstrated by the use of a mutant defective in envelope production but not viral DNA synthesis. Expression of such a mutant viral genome in primary hepatocytes allowed for the amplification of input viral DNA and enhanced production of viral gene products; this made it possible for us to detect small numbers of infected hepatocytes.

Such a mutant, pSPDHBV.1S, was constructed by inserting a synthetic double-stranded DNA linker between the *Kpn*I site at nucleotide 1290 and the *Xba*I site at nucleotide 1358. The linker contained three single-base substitutions of the wild-type sequence that created termination codons in the envelope open reading frame but did not change the amino acid coding in the overlapping polymerase open reading frame (Fig. 8A). When this DNA was introduced as a dimer-containing plasmid into HuH7 cells, wild-type levels of replicative forms of viral DNA were detected by Southern blot hybridization (Fig. 8B, compare lanes 1 and 2). In addition, accumulation of core antigen, but not surface antigen, could be seen by immunostaining of the fixed cells.

When supernatants of HuH7 cells transfected with the pSPDHBV.1S dimer DNA were used to infect primary duck hepatocyte cultures, no synthesis of viral DNA could be detected (Fig. 8B, lane 5). However, when mutant plasmid DNA was cotransfected with ΔDR1 into HuH7 cells and the culture fluids were used to infect hepatocytes, new viral DNA synthesis could be detected in the hepatocytes (lane 7). Immunostaining of the infected-cell layer (Fig. 9) revealed isolated cells that accumulated levels of core antigen as high as or higher than wild-type levels. No staining for envelope antigens could be detected in the mutant-infected cells, although envelope antigen accumulation was readily apparent in wild-type-infected cells. These experiments demon-

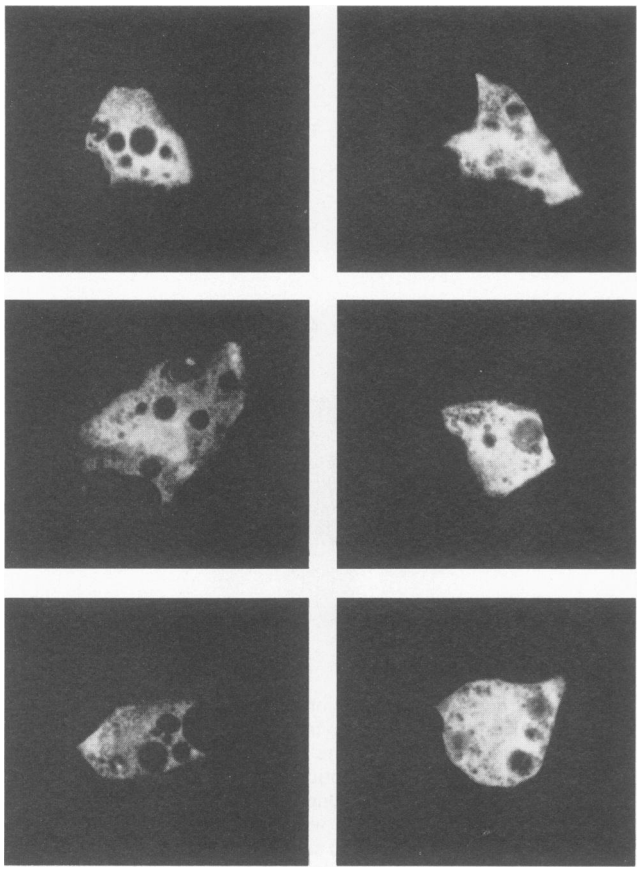

FIG. 9. Expression of core antigen in primary hepatocytes infected by wild-type and mutant genomes. Primary hepatocytes infected as in Fig. 8 were fixed 8 days postinfection and assayed by indirect immunofluorescence for core antigen. The figure shows examples of cells infected with wild-type virus (left-hand panels) or envelope mutant virus (right-hand panels).

strate that the ΔDR1 mutant provides in *trans* envelope functions required for infectivity of virus particles.

Suppression of virus production by cotransfection with K^- or K^-S^- genomes. Although defective genomes could be included in infection-competent particles by complementation in *trans*, some mutations apparently interfered with virus production from the wild-type genome. Plasmids containing defective genomes were cotransfected with the wild-type dimer plasmid into HuH7 cells, culture fluids were harvested after 5 days and placed on duck hepatocytes, and the hepatocytes were examined 10 days later for viral DNA. When cotransfection was carried out with either ΔDR1 dimer or the unoccupied vector pSP65, no suppression of infectious virus production was observed (Fig. 10). Thus, the presence of ΔDR1 apparently does not interfere with packaging, DNA synthesis, or infectious virus production by a second, cotransfected DHBV genome. In contrast with this lack of effect, when K^- or K^-S^- dimer genomes were cotransfected with the wild type, the production of virus by the HuH7 cells was strikingly reduced. In the case of K^-S^-, virus production was reduced about 30-fold. The defects engineered into these two genomes should affect viral gene products: the K^- genome is predicted to produce truncated pre-S1 and S products and a fusion product joining *pol* with S; the K^-S^- genome is predicted to produce these same altered products, as well as a truncated core product. These

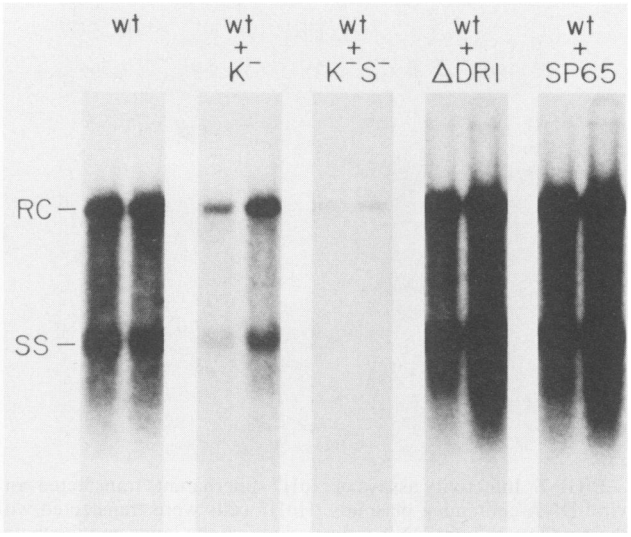

FIG. 10. Suppression of infectious virus production by cotransfection of mutant viral DNA-containing plasmids into HuH7 cells. HuH7 cells were transfected with 3 μg of each DNA per 60-mm dish. After a 5-day incubation, 1 ml of the 48-h culture fluids was each transferred onto two 60-mm dishes of primary hepatocytes for assay of infectivity as in Fig. 7. Total DNA was harvested at 10 days postinfection and analyzed for viral DNA by blot hybridization.

altered products may exert an effect on virus production by interference with proper assembly or function of normal wild-type-encoded counterparts.

DISCUSSION

Packaging and reverse transcription of replication-defective DHBV genomes. In this communication we report that replication-defective DHBV genomes can be packaged into virus particles and reverse transcribed into complete genomes when introduced into the human hepatoma cell line HuH7 along with a mutant DHBV genome deleted of the 12-base repeat called DR1. Three types of evidence indicate that the ΔDR1 genome acts as a helper by providing, in *trans*, functions required for packaging a second genome: (i) fluorescent staining directly demonstrated the expression from ΔDR1 of core and envelope structural proteins; (ii) cotransfection experiments showed that when transferred with the ΔDR1 genome, DHBV genomes defective in any or all of the three known viral genes could be complemented for the synthesis of packaged relaxed-circular viral DNA; and (iii) infection-competent particles from such cotransfections were able to transfer a viral infection to primary hepatocyte cultures. The identification of relaxed-circular DNA forms of the mutant or recombinant genomes themselves in viral core particles and the transfer of a mutant viral phenotype to hepatocyte cultures demonstrate that genetically defective viral DNAs can be rescued by complementation by ΔDR1.

Although the ΔDR1 genome expresses the viral functions required for packaging and DNA synthesis of a second defective genome, it is unable to direct the synthesis of a relaxed-circular double-stranded DNA copy of itself. Its pregenomic RNA is apparently packaged into particles, because we observed a small amount of minus-strand DNA transcript within particles when ΔDR1 was transfected alone, but we do not know the efficiencies of packaging or minus-strand DNA synthesis alone. At any rate, the ΔDR1

genome must contain at least part of a putative signal required for insertion of pregenomic RNA into particles. The synthesis of minus-strand material following ΔDR1 transfection came as a surprise to us because previous studies addressing the pathway of reverse transcription indicate that minus-strand synthesis commences at DR1. Recent examination of the ΔDR1-derived minus-strand species reveals that its 5′ terminus maps to a position in pregenomic RNA that is downstream from the site of deletion (W. S. Mason, personal communication). Thus, a cryptic or minor initiation site is apparently revealed by the deletion of DR1.

Although minus-strand synthesis from the ΔDR1 pregenome can proceed, albeit to a reduced extent, relaxed-circular DNA synthesis was not detected. Synthesis of relaxed-circular DNA requires plus-strand synthesis, which initiates at DR2 and uses a short RNA primer, apparently derived from the 5′ terminus of pregenomic RNA (8). This oligoribonucleotide includes the sequence DR1 at its 3′ end, which duplexes with the DR2 sequence present in the minus strand to position the site of initiation of plus-strand synthesis and act as a primer. We presume that in the absence of DR1, the production of a suitable primer cannot occur and, as a result, proper second-strand synthesis is inhibited.

In contrast to the ΔDR1 mutant, the rescuable defective genomes described here contain intact copies of both DR1 and DR2, and viral DNA synthesis with these pregenomic RNAs as templates should proceed normally. Despite the presence of both repeats, however, the levels of packaged relaxed-circular forms of these genomes were variable and, in general, appeared to be lower than those observed after transfection of a wild-type genome. Potential explanations for this result include differences in the transient expression of plasmids that carry different vector backbones (pBR322 versus pSP65); a negative effect of deletion of four residues from the ΔDR1-generated precore protein; or a possible preference for one or more viral gene products to function in *cis* to allow full efficiency of packaging or DNA synthesis.

The cotransfection studies also revealed a possible constraint on the size of the defective genome that can be packaged and reverse transcribed or a constraint on the location at which foreign sequences can be inserted. A recombinant DHBV genome only 5% larger than the wild-type genome was rescued at low efficiency, and a similar recombinant genome 30% larger than the wild type was not rescued at the limit of our detection. In contrast, a 4-base-pair deletion at the site of insertion of foreign sequences had little effect on rescue. If the factor affecting the efficiency of rescue is the size of the recombinant pregenome, it is important to determine how much of the DHBV genome can be replaced with foreign sequences in an attempt to assess the suitability of hepadnavirus-based vectors for gene transfer.

Production of infectious virus by HuH7 cells. Previous studies have shown that infectious virus was released into the supernatant of HuH7 cells transfected with a wild-type DHBV genome. This system allowed us to confirm that transfection of HuH7 cells with various mutant viral DNAs alone did not result in the production of infectious virus. Moreover, in attempts to detect the transfer of mutant viral DNA genomes that were defective in DNA replication (RV 2650 and Kpn^-), we have been unable to detect the production of viral replicative intermediates or viral antigens, which indicates that recombination of mutant genomes with ΔDR1 to produce wild-type virus occurs, if at all, at levels below our limit of detection. However, when a mutant capable of viral DNA synthesis but defective in envelope proteins was cotransfected with ΔDR1 into HuH7 cells, particles capable of inducing viral DNA synthesis in hepatocytes were produced. The detection of core antigen-staining cells, but not surface antigen-staining cells, indicates that the DNA synthesis observed was due to transfer of the mutant genome. We do not believe that this mutant genome is uniquely transferred among the mutants we have tested, but we believe that transfer is detectable only because of increased levels of the intracellular viral DNA template (cccDNA), as well as the ability to produce replicative intermediates. We are currently in the process of testing hepatocytes infected by this mutant genome for amplification of viral cccDNA.

We could estimate from this experiment that 10^3 to 10^4 hepatocytes were infected by 1 ml of culture fluid. This low level of virus production (at least 10-fold below that produced from wild-type-transfected cells) might be a consequence of the expression by the mutant of a truncated envelope protein which interferes with the assembly of functional envelopes. Examples of such interfering effects were observed with two mutant genomes.

Cotransfection of either of the mutant viral DNAs, K^- and K^-S^-, with wild-type DNA suppressed the production of infectious wild-type virus by HuH7 cells as assayed in primary hepatocytes. Two comments of interest may be made concerning this observation. First, since cotransfection of the wild type with K^-S^- DNA reduced the yield of virus at least 30-fold, more than 97% (and probably 100%) of the cotransfected cells must be expressing both transfected genomes. Therefore, the somewhat low efficiencies of complementation of DNA synthesis by ΔDR1 cannot be explained by a low efficiency of cotransformation.

Second, interference by mutants of wild-type virus production may represent the effects of truncated or defective viral proteins in competing with wild-type proteins for the formation of multisubunit structures, such as nucleocapsids and envelopes. The K^-S^- mutant can direct the synthesis of a polypeptide consisting of 67 NH_2-terminal amino acids of the major core protein and 110 NH_2-terminal residues of the pre-core. That one or both of these products may interfere with the assembly of wild-type nucleocapsid protein into stable structures is indicated by the ability of the mutant DNA, when cotransfected with the wild type, to eliminate detectable staining for core antigen in immunofluorescence assays (data not shown). Like the K^- mutant, the double mutant K^-S^- introduces a frameshift and subsequent termination into the envelope (pre-S1-S) open reading frame at amino acid 199, as well as a shift in the polymerase open reading frame at amino acid 330, into the remainder of the envelope open reading frame. Both of the mutants suppress the expression of envelope antigen when cotransfected into HuH7 cells with wild-type DNA, as assayed by immunofluorescent staining (data not shown), suggesting an interference with the assembly of wild-type envelope proteins into stable structures. In our experiments, such interference may substantially contribute to the low level of mutant genome rescue observed.

Interference by an altered product with the normal function of a viral protein has been reported for a transcriptional activator protein of herpes simplex virus type 1, VP16 (3). Both in that case and in the example presented here, the phenomenon of a dominant negative phenotype may eventually be used therapeutically to inhibit virus production in naturally occurring infections.

Although the experiments presented here indicate the feasibility of producing replication-defective HBVs, further study is required to address whether these particles are

capable of efficiently transducing hepatic cell populations. If transduction can be achieved, questions will have to be addressed concerning the maintenance and expression of defective genomes and concerning the safety of the host. These questions should be approachable by using the duck system as a model.

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