JOURNAL OF VIROLOGY, Feb. 1992, p. 1282–1287
0022-538X/92/021282-06$02.00/0

Vol. 66, No. 2

Naturally Occurring Point Mutation in the C Terminus of the Polymerase Gene Prevents Duck Hepatitis B Virus RNA Packaging

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A duck hepatitis B virus (DHBV) genome cloned from a domestic duck from the People's Republic of China has been sequenced and exhibits no variation in sequences known to be important in viral replication or generation of gene products. Intrahepatic transfection of a dimer of this viral genome into ducklings did not result in viremia or any sign of virus infection, indicating that the genome was defective. Functional analysis of this mutant genome, performed by transfecting the DNA into a chicken hepatoma cell line capable of replicating wild-type virus, indicated that viral RNA is not encapsidated. However, virus core protein is made and can assemble into particles in the absence of encapsidation of viral nucleic acid. Using genetic approaches, it was determined that a change of cysteine to tyrosine in position 711 in the polymerase (P) gene C terminus led to this RNA-packaging defect. By site-directed mutagenesis, it was found that while substitution of Cys-711 with tryptophan also abolished packaging, substitution with methionine did not affect packaging or viral replication. Therefore, Cys-711, which is conserved in all published sequences of DHBV, may not be involved in a disulfide bridge structure essential to viral RNA packaging or replication. Our results, showing that a missense mutation in the region of the DHBV polymerase protein thought to be primarily the RNase H domain results in packaging deficiency, support the previous findings that multiple regions of the complex hepadnaviral polymerase protein may be required for viral RNA packaging.

Hepadnaviruses are a small group of enveloped DNA viruses which consist of human, duck, ground squirrel, woodchuck, and heron hepatitis B viruses (4, 19, 20, 28, 31). The major steps thought to be fundamental to the replication of hepadnaviral genomes are (i) conversion of virion relaxed-circular DNA to covalently closed circular (CCC) DNA; (ii) transcription of CCC DNA to generate pregenomic RNA; (iii) encapsidation of pregenomic RNA and products of the P gene into virus cores or nucleocapsids; (iv) synthesis by reverse transcription of the first (minus) strand of DNA using the P gene-encoded protein primer (terminal protein); and (v) synthesis of the second (plus) strand of DNA using an oligomer of viral RNA as a primer (6, 12). In the encapsidation or packaging reaction, viral proteins specifically recognize and bind the viral pregenome and each other. To date, little is known about the molecular mechanisms involved in this process. A short sequence located near the 5′ end of pregenomic RNA has recently been identified as the signal important for encapsidation of HBV pregenomic RNA (1, 17). Viral core protein, the product of the C gene, is the principal structural protein of the viral nucleocapsid (12). There is recent evidence that the product of the P gene is required in addition to that of the C gene for virus RNA encapsidation (1, 15).

In this study, we have analyzed a naturally occurring defective duck hepatitis B virus (DHBV) DNA cloned in bacteria by observing its properties in a transient transfection system in which infectious virus can be produced (11, 30). We have found that the defective DHBV genome was unable to encapsidate viral RNA, although it produced adequate amounts of both core protein and viral pregenomic RNA. The lesion has been localized to a change in a single amino acid residue in the carboxy terminus of the P protein.

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The DHBV22 genome is defective in encapsidating viral RNA into cores. DHBV22 is a viral DNA cloned from virus from the serum of a duck from Qidong county in the People's Republic of China. Sequencing of DHBV22 DNA did not reveal any variation in sequences known to be important in viral replication or generation of gene products (29). Intrahepatic transfection of this viral genome into susceptible Pekin ducklings did not result in infection, in contrast to control transfection of wild-type (DHBV16) DNA (19a).

To investigate the replication defect in DHBV22, we tested the ability of this mutant to synthesize viral DNA, transcripts, and proteins. A head-to-tail dimer of DHBV16, a functional genome, cloned into the plasmid pGEM-4 *Eco*RI site (25) and a similar construct of the mutant genome DHBV22 were transfected into LMH cells as described previously (8). Three to five days posttransfection, core particles were isolated from cells transfected with each DNA, and the DNAs were purified from the particles (25) and analyzed by Southern blotting (33). The results are shown in Fig. 1A. Cells receiving the DHBV16 genome (in plasmid pGEM-DHBV16) contained both single-stranded viral DNA and relaxed-circular DNA, i.e., the replicating and mature viral DNA forms, respectively (Fig. 1A, lane 1). In contrast, cells transfected with the DHBV22 genome (in plasmid pGEM-DHBV22) did not express any replicating and maturing viral DNA in core particles (lane 2). Thus, the transfected DHBV22 genome failed to be converted into a packaged single-stranded or relaxed-circular DNA molecule.

The limit of DHBV22 replication did not appear to be the result of a drastically reduced level of viral RNA. When the total poly(A)$^+$ virus-specific RNA (14) from transfected cells was examined 2 days after transfection with either DHBV16 or DHBV22, two major classes of virus-related transcripts corresponding to genomic (3.3 kb) and subgenomic (1.9 and 2.0 kb) RNA were detected at approximately the same levels (data not shown). The levels of core proteins and surface

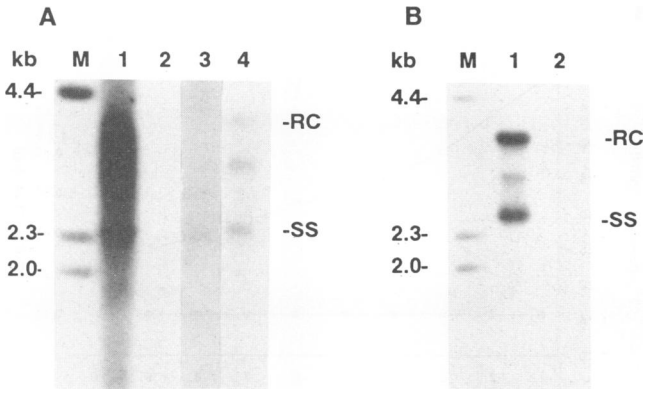

FIG. 1. (A) DNA replication of the DHBV16 and DHBV22 genomes and transcomplementation analysis of pGEM-DHBV22 and ΔDR1. Southern blot analysis of cytoplasmic DNA isolated from LMH cells at 5 days posttransfection. LMH cells were grown on 6-well plates under previously described conditions (11). DNA transfections were performed with 5 μg of plasmid DNA per well of nearly confluent cells. Kodak XAR film was exposed to the filter at −70°C for 2 days. Lanes: 1, pGEM-DHBV16; 2, pGEM-DHBV22; 3, ΔDR1; 4, pGEM-DHBV22 and ΔDR1; M, ^{32}P-labelled DNA size markers from a *Hin*dIII digest of bacteriophage lambda. Lane 3 was overexposed to allow detection of the small amount of single-stranded viral DNA. (B) DNA replication after transfection with pGEM-DHBV16 or pGEM-DHBV16-2301 DNAs. Southern blot analysis of cytoplasmic DNA isolated from LMH cells at 5 days posttransfection. Film was exposed to the filter overnight at −70°C. Lanes: 1, pGEM-DHBV16; 2, pGEM-DHBV16-2301; M, ^{32}P-labelled DNA size markers from a *Hin*dIII digest of bacteriophage lambda. RC indicates the position of relaxed-circular DHBV DNA, and SS indicates single-stranded DHBV DNA.

proteins in LMH cells transfected with DHBV22 were identical to those seen in DHBV16-transfected cells by using immunofluorescence staining (33) (data not shown), showing that the viral transcripts were able to serve as mRNA for the synthesis of these antigens. No infectious virus was produced by LMH cells transfected with DHBV22 (data not shown).

To determine whether DHBV22 viral RNA was encapsidated into core particles, cytoplasmic extracts of cells taken from three 6-well plates 2 days after transfection were layered onto 8 ml of 10% sucrose in TNE (18) and centrifuged at 68,000 × *g* for 24 h at 4°C. The supernatant was removed, and the pellet was suspended in TNE buffer. CsCl was added to yield a solution with a density of 1.35 g/ml, and the samples were subjected to isopycnic centrifugation at 200,000 × *g* for 24 h at 10°C. Four hundred microliters of each fraction was collected from the bottom of the tube, and the density was determined with a refractometer. A 40-μl sample from each fraction was analyzed by Northern (RNA) slot blotting by using a ^{32}P-labelled DHBV antisense RNA probe (23). The results shown in Fig. 2A indicate that in cells transfected with DHBV16, viral RNA is present in core particles at a density of 1.34 g/ml, similar to results obtained in previous studies of core particles (21, 32). However, no viral RNA was detected at this density or at any other density in the extracts of cells transfected with DHBV22 (Fig. 2B). These studies indicate either that no core particles are made by DHBV22 or that little or no viral RNA is encapsidated.

To investigate whether core particles were produced by DHBV22, samples of CsCl gradient fractions were analyzed by Western immunoblotting (9, 10) using antibody made to DHBV core (34). The results of this experiment are shown in Fig. 2C and D. In cells transfected with the replication-competent DHBV16, core proteins were present in a peak at a density of 1.34 g/ml, which is that of nucleocapsids containing DHBV DNA (24a). In cells transfected with DHBV22, core protein was detected as a peak at a lower density (1.30 g/ml), indicating that core particles are present in the DHBV22 cell lysate but are most likely empty (22, 24, 32).

A change of Cys to Tyr at amino acid residue 711 in the carboxy terminus of the P gene product leads to the encapsidation deficiency. To determine which nucleotide sequence in DHBV22 confers viral RNA encapsidation deficiency, we made a series of chimeric dimers of DHBV16 and DHBV22. Each chimeric dimer was transfected into LMH cells, and the replicating and mature viral DNA was detected by Southern blotting (data not shown). Detection of viral DNA in this fashion can be used as an indirect measurement to test pregenome RNA encapsidation, as it tests for a step in the life cycle which is dependent upon prior RNA encapsidation (1). This study indicated that the sequences related to the encapsidation deficiency were found between nt 2184 and 2352 of the second monomer of the pGEM-DHBV22 genome. A comparison of DHBV nucleotide sequences of published DHBV genomes cloned from ducks from the People's Republic of China, the United States, and Europe (26a) indicated that two nucleotide changes in this region were unique to DHBV22, one at nt 2301 (G to A) and the other at nt 2236 (C to T). Only the first change results in an amino acid change, in which the cysteine at amino acid residue 711 of the P protein is replaced with a tyrosine. This cysteine is conserved in all the other cloned DHBV DNAs, though not in all hepadnaviruses, and is located in the carboxy-terminal region of the P protein.

To determine whether the encapsidation deficiency of DHBV22 can be rescued by a viral genome which expresses a functional P protein, we tested the ability of the ΔDR1 mutant to complement DHBV22. This mutant has 12 nucleotides deleted in the DR1 region and provides all viral proteins in *trans*, but is itself defective in production of packaged relaxed-circular viral genome (16). When DHBV22 and the ΔDR1 genome were cotransfected into LMH cells, relaxed-circular viral DNA was detectable in core particles (Fig. 1A, lane 4), and only a very small amount of minus-strand DNA was seen when ΔDR1 was transfected alone into LMH cells (Fig. 1A, lane 3). This result indicates that the encapsidation deficiency of DHBV22 can be overcome by P protein supplied in *trans*.

We next focused on the change at nt 2301 which leads to a missense mutation and generated a site-specific mutation of DHBV16 in which the G at nt 2301 was changed to A. By using oligonucleotides containing the desired sequences, the fragment was amplified by PCR and cloned back into pGEM-DHBV16 (13), which was then used to make a head-to-tail dimer to test for viral DNA replication and encapsidation. Figure 1B shows that no viral DNA replication was detectable after the missense mutant pGEM-DHBV16-2301 was transfected into LMH cells (lane 2), although immunofluorescence staining showed that pGEM-DHBV16-2301 strongly expressed core and surface antigens (data not shown), indicating that this mutant can synthesize normal levels of viral transcripts. No encapsidated viral RNA from DHBV16-2301 was detected in nucleocapsids by Northern slot blot analysis, in contrast to DHBV16 nucleocapsid RNA analysis (Fig. 3A), although similar amounts of core particles

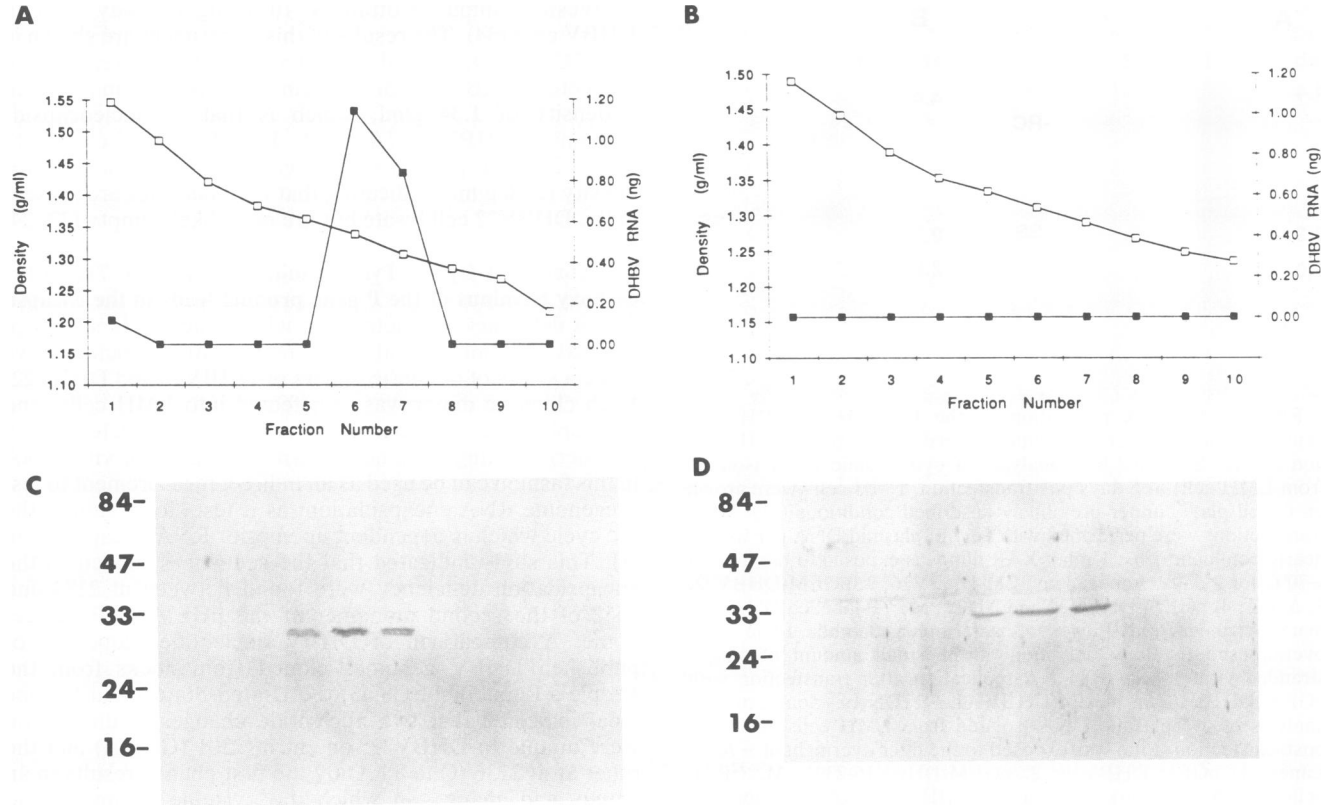

FIG. 2. Detection of DHBV nucleocapsids in LMH cells transfected with pGEM-DHBV16 (A and C) or pGEM-DHBV22 (B and D) DNAs. Cells lysed at 2 days posttransfection were subjected to isopycnic centrifugation in CsCl. Gradient densities are shown by the open squares. Viral RNA, shown by the filled squares (A and B), was detected by Northern slot blotting (23). Core protein (C and D) was detected by Western blotting. Lanes containing material from individual fractions are located beneath the appropriate fraction number.

were present in both cytoplasmic lysates (Fig. 3B). These data confirm that changing cysteine to tyrosine at amino acid residue 711 in the carboxy terminus of the P protein prevents packaging of DHBV RNA into virus particles.

Cys-711 may not be in a disulfide bridge structure. To better understand the involvement of Cys-711 in viral packaging, two additional missense mutants were made: pGEM-16-711M, in which Cys-711 was changed to methionine, and pGEM-16-711W, in which tryptophan was substituted for Cys-711. These amino acids were tested for packaging activity because methionine is similar in size to cysteine and also has a sulfur-containing side chain, yet gives an altered predicted P product structure when substituted for Cys-711 (Chou-Fasman structure prediction not shown) and because tryptophan bears size and side chain similarity to tyrosine but does not result in the predicted altered P protein structure seen when tyrosine is substituted for Cys-711. When the mutants were transfected into LMH cells, immunofluorescence staining showed that both strongly expressed core and surface antigens (data not shown), indicating that the mutants can synthesize normal levels of viral transcripts. We then tested (by Southern blotting) viral replication of these two mutants after transfection into LMH cells (Fig. 4). While a pattern of replicating viral DNA was present in core particles from cells transfected with the mutant pGEM-16-711M (lane 2), no detectable viral DNA replicating forms were seen in core particles from cells transfected with the mutant pGEM-16-711W (lane 3). In this experiment, in which cells were lysed at 3 days posttransfection, the predominant replicating viral DNA form was single stranded, in contrast to the pattern seen in Fig. 1, obtained with cells harvested at 5 days after transfection. This mutational study indicates that computer structural analyses were less predictive of P protein encapsidation function at this amino acid residue than were similarity in size or side chain. Moreover, the ability of methionine to substitute for Cys-711 to allow packaging and replication indicates that Cys-711 is unlikely to be involved in a disulfide bridge structure essential to viral RNA packaging or replication.

From the results of mutational and theoretical structural analyses of the P protein, as well as sequence comparison with other genes producing reverse transcriptases, the hepadnavirus P product has been divided into the following four domains, listed from the N terminus to the C terminus: the terminal protein thought to serve as primer of the minus DNA strand, a spacer region, the reverse transcriptase, and the RNase H domain (2). Genetic studies of both HBV and DHBV P genes indicate that frameshift mutations throughout the P gene were defective for viral RNA encapsidation (1, 15). Bartenschlager and colleagues found that in-frame deletions of all four P protein domains also led to a drastic reduction in packaging efficiency of HBV (1). Blum and coworkers have recently reported a missense mutation in the terminal protein domain of the HBV polymerase protein which results in an inability to package viral RNA (3). Our missense mutation is located at the opposite end of the

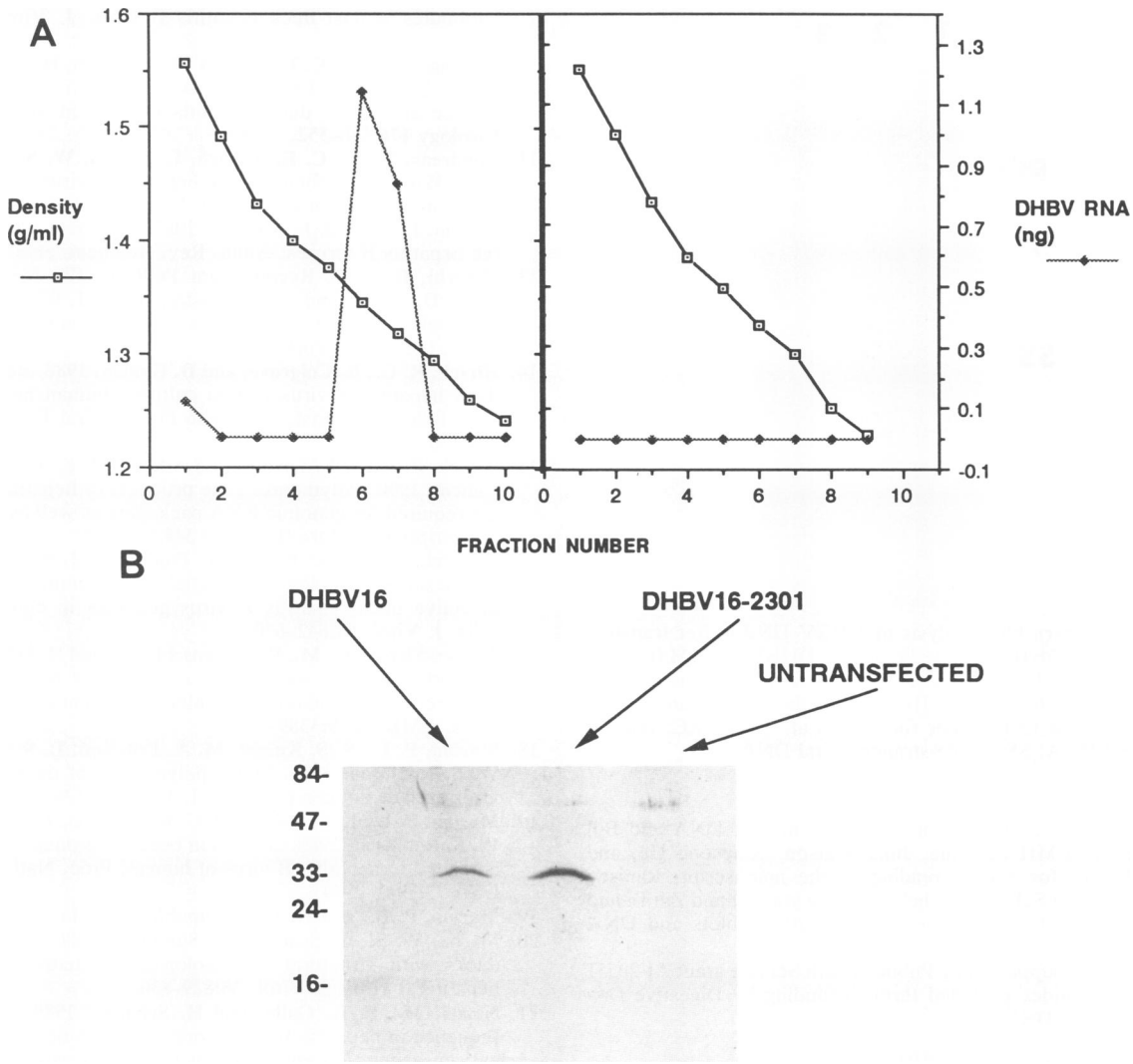

FIG. 3. Detection of encapsidated DHBV RNA and core proteins in viral particles from LMH cells transfected with pGEM-DHBV16 and pGEM-DHBV2301. (A) Cells lysed at 2 days posttransfection were subjected to isopycnic centrifugation in CsCl. Viral RNA in the fractions was detected by Northern slot blotting. (B) Particulate core proteins detected by Western blotting.

DHBV P gene, in the RNase H domain. These data support the notion that multiple regions of the P protein are required for RNA encapsidation by hepadnaviruses (1, 7).

The specific requirement for the P gene product in the encapsidation process suggests the possibility that this protein may provide for the specific recognition of the pregenomic RNA, possibly at the RNA encapsidation signal, or at the signal for reverse transcription (27), which is 3 kb away, or at both locations (1). The P protein of DHBV appears to bind firmly to nucleocapsids found in natural infection (26). This binding, in turn, could be due to a specific binding of the P protein to viral nucleic acid which is bound to the capsid proteins, a specific binding of the P protein to the capsid proteins, or a combination of the two.

At least two possibilities might explain why a missense mutation in the reverse transcriptase/RNAse H portion of the P protein prevents packaging. First, the mutation may affect the stability of this protein, which may exist and function as a large uncleaved molecule (5), and lead to its rapid degradation. As it was not possible for us to examine directly the turnover of this protein in transfected cells, we turned to the use of limited proteolytic digestion of mutant and wild-type P proteins made by in vitro translation (data not shown) but were unable to detect a difference in the rate of appearance of trypsin fragments of the mutant compared with that of the wild type. This result suggests that the missense mutation does not alter the protein conformation to the extent that normally protected trypsin cleavage sites are made accessible. In the cell, however, this mutant protein may still be more quickly degraded than its normal counterpart. A second possible reason for the lack of packaging with the mutant protein is that it may be unable to interact with the viral RNA or the capsid proteins, either because of a conformational change in which the specific recognition sites are altered or because this amino acid residue is involved in specific recognition. Additional site-directed mutagenesis studies will be necessary to determine whether Cys-711 may be part of a recognition signal for packaging of DHBV RNA.

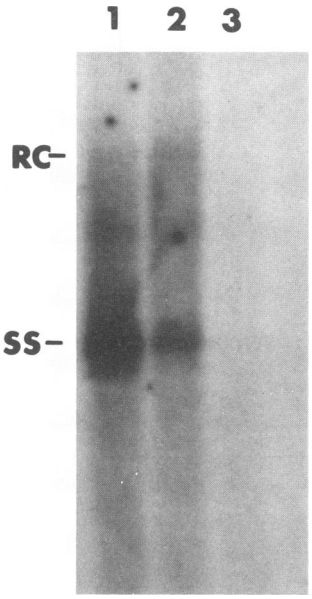

FIG. 4. Southern blot analysis of DHBV DNAs after transfection with pGEM-DHBV16 (lane 1), pGEM-DHBV16-711M (lane 2), and pGEM-DHBV16-711W (lane 3) DNAs. Analysis was of cytoplasmic DNA isolated from LMH cells at 3 days after transfection. Film was exposed to the filter for 2 days at −70°C. RC, relaxed circular viral DNA; SS, single-stranded viral DNA.

We thank Jesse Summers for the ΔDR1 mutant DNA and Bill Mason for the LMH cell line, Julia Watson, Xiaosong He, and Averell Sherker for careful reading of the manuscript, Ramsey Cheung and Marci Schaner for help with the graphs, and Zili Li and Irina Cross for help with some of the Southern blots and DNA sequencing.

This work was supported by Public Health Service grant AI-20551 with oligonucleotides provided through funding by Digestive Diseases Core Grant DK38707.

REFERENCES

1. **Bartenschlager, R., M. Junker-Niepmann, and H. Schaller.** 1990. The P gene product of hepatitis B virus is required as a structural component for genomic RNA encapsidation. J. Virol. **64**:5324–5332.
2. **Bartenschlager, R., and H. Schaller.** 1988. The amino-terminal domain of hepadnaviral P-gene encodes the terminal protein (genome-linked protein) believed to prime reverse transcription. EMBO J. **7**:4185–4192.
3. **Blum, H. E., E. Galun, T. J. Liang, F. V. Werzsaecker, and J. R. Wands.** 1991. A naturally occurring missense mutant in the polymerase gene terminating hepatitis B virus replication. J. Virol. **65**:1836–1842.
4. **Blumberg, B. S., H. J. Alter, and S. Visnich.** 1965. A 'new' antigen in leukaemia sera. JAMA **191**:541–546.
5. **Bosch, V., R. Bartenschlager, G. Radziwill, and H. Schaller.** 1988. The duck hepatitis B virus P-gene codes for a protein strongly associated with the 5'-end of the minus strand. Virology **166**:475–485.
6. **Cattaneo, R., H. Will, and H. Schaller.** 1984. Hepatitis B virus transcription in infected liver. EMBO J. **3**:2191–2196.
7. **Chang, L.-J., D. Ganem, and H. E. Varmus.** 1990. Effects of insertional and point mutations on the functions of duck hepatitis B virus polymerase. J. Virol. **64**:5553–5558.
8. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7**:2745–2752.
9. **Cheung, R. C., W. S. Robinson, P. L. Marion, and H. B. Greenberg.** 1989. Epitope mapping of neutralizing monoclonal antibodies against duck hepatitis B virus. J. Virol. **63**:2445–2451.
10. **Cheung, R. C., D. E. Trujillo, W. S. Robinson, H. B. Greenberg, and P. L. Marion.** 1990. Epitope-specific antibody response to surface antigen of duck hepatitis B virus in infected ducks. Virology **176**:546–552.
11. **Condreay, L. D., C. E. Aldrich, L. Coates, W. S. Mason, and T. T. Wu.** 1990. Efficient duck hepatitis B virus production by an avian liver tumor cell line. J. Virol. **64**:3249–3258.
12. **Ganem, D., and H. E. Varmus.** 1987. The molecular biology of the hepatitis B viruses. Annu. Rev. Biochem. **56**:651–693.
13. **Higuchi, R.** 1990. Recombinant PCR, p. 177–183. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, Inc., New York.
14. **Hirsch, R. C., R. Colgrove, and D. Ganem.** 1988. Replication of duck hepatitis B virus in two cultured human hepatoma cell lines following transfection with cloned viral DNA. Virology **167**:136–142.
15. **Hirsch, R. C., J. E. Lavine, L.-J. Chang, H. E. Varmus, and D. Ganem.** 1990. Polymerase gene products of hepatitis B viruses are required for genomic RNA packaging as well as for reverse transcription. Nature (London) **344**:552–555.
16. **Horwich, A. L., K. Furtak, J. Pugh, and J. Summers.** 1990. Synthesis of hepadnavirus particles that contain replication-defective duck hepatitis B virus genomes in cultured HuH7 cells. J. Virol. **64**:642–650.
17. **Junker-Niepmann, M., R. Bartenschlager, and H. Schaller.** 1990. A short cis-acting sequence is required for hepatitis B virus pregenome encapsidation and sufficient for packaging of foreign RNA. EMBO J. **9**:3389–3396.
18. **Marion, P. L., S. S. Knight, M. A. Feitelson, L. S. Oshiro, and W. S. Robinson.** 1983. Major polypeptide of duck hepatitis B virus surface antigen particles. J. Virol. **48**:534–541.
19. **Marion, P. L., L. S. Oshiro, D. C. Regnery, G. H. Scullard, and W. S. Robinson.** 1980. A virus in beechey ground squirrels that is related to hepatitis B virus of human. Proc. Natl. Acad. Sci. USA **77**:2941–2944.

19a. **Marion, P. L., and H. Will.** Unpublished data.

20. **Mason, W. S., G. Seal, and J. Summers.** 1980. Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. J. Virol. **36**:829–836.
21. **Nassal, M., P. R. Galle, and H. Schaller.** 1989. Proteaselike sequence in hepatitis B virus core antigen is not required for e antigen generation and may not be part of aspartic acid-type protease. J. Virol. **63**:2598–2604.
22. **Ou, J.-H., and K. Bell.** 1990. Comparative studies of hepatitis B virus precore and core particles. J. Virol. **174**:185–191.
23. **Paeratakul, U., P. R. De Stasio, and M. W. Taylor.** 1988. A fast and sensitive method for detecting specific viral RNA in mammalian cells. J. Virol. **62**:1132–1135.
24. **Petit, M.-A., and J. Pillot.** 1985. HBc and HBe antigenicity and DNA-binding activity of major core protein p22 in hepatitis B virus core particles isolated from the cytoplasm of human liver cells. J. Virol. **53**:543–551.

24a. **Pugh, J., A. Zweidler, and J. Summers.** 1989. Characterization of the major duck hepatitis B virus core particle protein. J. Virol. **63**:1371–1376.

25. **Pugh, J. C., K. Yaginuma, K. Koike, and J. Summers.** 1988. Duck hepatitis B virus (DHBV) particles produced by transient expression of DHBV DNA in a human hepatoma cell line are infectious in vitro. J. Virol. **62**:3513–3516.
26. **Radziwill, G., H. Zentgraf, H. Schaller, and V. Bosch.** 1988. The duck hepatitis B virus DNA polymerase is tightly associated with the viral core structure and unable to switch to an exogenous template. Virology **163**:123–132.

26a. **Schödel, F., T. Weimer, D. Fernholz, R. Schneider, R. Sprengel, G. Wildner, and H. Will.** 1991. The biology of avian hepatitis B viruses, p. 53–80. *In* A. McLachlan (ed.), Molecular biology of the hepatitis B viruses. CRC Press, Boca Raton, Fla.

27. **Seeger, C., and J. Maragos.** 1990. Identification and characterization of the woodchuck hepatitis virus origin of DNA replication. J. Virol. **64**:16–23.

28. **Sprengel, R., E. F. Kaleta, and H. Will.** 1988. Isolation and characterization of hepatitis B virus endemic in herons. J. Virol. **62**:3832–3839.
29. **Sprengel, R., R. Schneider, P. L. Marion, D. Fernholz, G. Wildner, and H. Will.** 1991. Comparative sequence analysis of defective and infectious avian hepadnaviruses. Nucleic Acids Res. **19**:4289.
30. **Summers, J., P. M. Smith, and A. L. Horwich.** 1990. Hepadnavirus envelope protein regulate covalently closed circular DNA amplification. J. Virol. **64**:2819–2824.
31. **Summers, J., J. M. Smolec, and R. Snyder.** 1978. A virus similar to hepatitis B virus associated with hepatitis and hepatoma in woodchucks. Proc. Natl. Acad. Sci. USA **75**:4522–4537.
32. **Sureau, C., J.-L. Romet-Lemonne, J. I. Mullins, and M. Essex.** 1986. Production of hepatitis B virus by a differentiated human hepatoma cell line after transfection with cloned circular HBV DNA. Cell **47**:37–47.
33. **Tagawa, M., W. S. Robinson, and P. L. Marion.** 1987. Duck hepatitis B virus replication in the yolk sac of developing embryos. J. Virol. **61**:2273–2279.
34. **Trujillo, D., and P. L. Marion.** Unpublished data.