JOURNAL OF VIROLOGY, Nov. 1989, p. 4965–4968
0022-538X/89/114965-04$02.00/0

Vol. 63, No. 11

Duck Hepatitis B Virus Can Tolerate Insertion, Deletion, and Partial Frameshift Mutation in the Distal Pre-S Region

JI-SU LI,[1] LUCYNA COVA,[1]* ROBIN BUCKLAND,[2] VÉRONIQUE LAMBERT,[1] GILBERT DELÉAGE,[3] AND CHRISTIAN TRÉPO[1]

Unité de Recherche sur les Hepatites, Institut National de la Santé et de la Recherche Médicale U 271, 69424 Lyon Cedex 03,[1] Laboratoire d'Immunovirologie Moleculaire et Cellulaire, Centre National de la Recherche Scientifique UMR 005, 69008 Lyon,[2] and Laboratoire de Physicochimie Biologique, LBTM Centre National de la Recherche Scientifique UMR 9, 69622 Villeurbanne Cedex,[3] France

Received 12 April 1989/Accepted 20 July 1989

In-frame and frameshift mutations were introduced into the pre-S region (1,212 base pairs) of duck hepatitis B virus. The in-frame mutants retained the inserted 12 nucleotides, while the frameshift mutants either reverted to wild type or exhibited a 10-nucleotide compensatory deletion downstream of the original mutation site. Thus, although duck hepatitis B virus has a compact and highly economical genome organization, it can replicate despite alterations of up to 9 amino acid codons in the pre-S and P open reading frames.

The human hepatitis B virus (HBV) is the prototype of hepadnaviruses. Related viruses are also found in woodchucks, ground squirrels, ducks, and herons (20, 22). The genomes of these viruses contain four open reading frames, namely S, C, P, and X. The S open reading frame is further divided into pre-S1, pre-S2, and S genes. Since the pre-S encoded sequence is highly variable among hepadnaviruses, it has been proposed to be involved in species-specific recognition of target cells (18). The pre-S proteins possibly contain a dominant binding site for hepatocytes (11) and can induce high-titer neutralizing antibodies (9, 10). The successful transfection of ducklings with cloned duck HBV (DHBV) DNA (21) facilitates direct mutational analysis of the entire pre-S region, making possible the definition of a receptor-binding site(s) as well as potential nonessential sequences. In this report, we have studied the effect of in-frame and frameshift mutations in the distal pre-S region of DHBV on viral replication capacity.

The construction of mutant DHBV clones is summarized in Fig. 1. Briefly, DHBV DNA (7) ligated into pBR322 was digested with *Xho*I, which cut at position 1212 of the pre-S region. The linearized DNA was either (i) ligated to a single-stranded linker TCGAAT (Pharmacia) and recircularized, giving an in-frame insertion of six (or a multiple of six) nucleotides (nt), or (ii) blunt ended by using DNA polymerase I (Klenow fragment) and religated, thus generating a +1 frameshift in the succeeding coding sequences of both pre-S and DNA polymerase genes. Both in-frame and frameshift mutant DNAs lost the original *Xho*I site and generated a *Eco*RI site and a *Pvu*I site, respectively. After transformation of *Escherichia coli* 1106 (8), the mutant DHBV DNA clones were extracted by the alkaline lysis method (8) and identified by digestion with *Eco*RI, *Xho*I, and *Pvu*I. To transfect ducklings, recircularized DHBV genome and the pBR322 integrated tandem dimer version were constructed as described by Sprengel et al. (21).

Mutant DHBV DNA (25 or 50 μg) was dissolved in 0.2 ml of Dulbecco essential medium containing 50 μg of DEAE-dextran and injected percutaneously into the livers of 2-day-old DHBV-free ducklings. A wild-type DHBV clone was also transfected into ducklings as a control. After transfection, ducks were bled and 50 μl of sera was tested for the presence of DHBV DNA by dot hybridization as described previously (2). Viremia was observed in 4 of 5 (80%) of the ducklings which received the in-frame mutant DNA but also in 7 of 25 (28%) of those transfected with the frameshift DNA (Fig. 2 and Table 1). In most mutant DNA-transfected ducklings, viremia was transient, as compared with animals transfected with wild-type DHBV DNA. Southern blotting with a ^{32}P-labeled DHBV DNA probe detected large quantities of the replicative forms of viral DNA in the livers of all viremic ducklings (data not shown). In contrast to the transient nature of the viremia, viral DNA in mutant DNA-transfected livers could be detected for as long as in wild-type DNA-transfected livers, i.e. up to 5 months posttransfection. Furthermore, the DHBV-positive sera were able to induce viremia when injected intravenously into DHBV-free ducklings (data not shown).

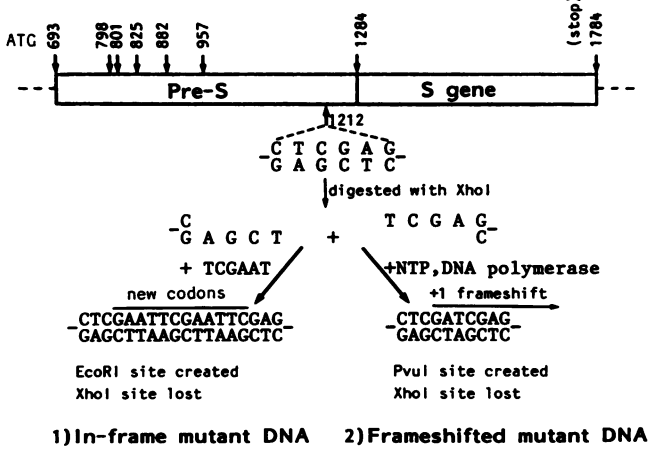

FIG. 1. Construction of mutant DHBV DNAs. The DHBV DNA envelope gene diagrammed at the top is based on the sequence of Mandart et al. (5). The positions of the in-frame ATGs and stop codon are indicated by arrows. The construction of in-frame and frameshift mutant DNAs at position 1212 of the pre-S region are shown below. The insertions of 12 nt in the in-frame mutant DNA and 4 nt in the frameshift mutant DNA used for transfection were confirmed by sequence analysis.

* Corresponding author.

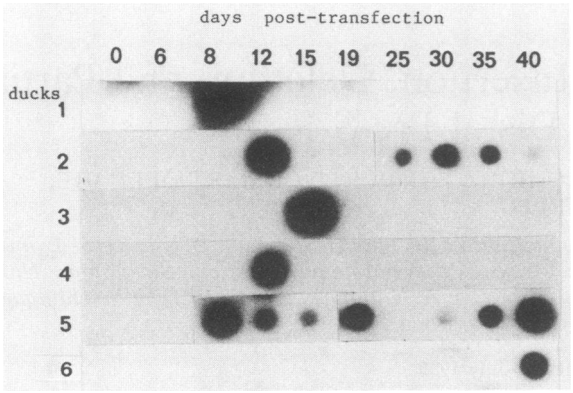

FIG. 2. Dot blot analysis of DHBV DNA in transfected duck sera. Ducks 1 and 2 were transfected with in-frame mutant DNA; ducks 3 and 4 were transfected with frameshift mutant DNA; duck 5 was transfected with wild-type DHBV DNA (control). Lane 6 contains DHBV-positive sera.

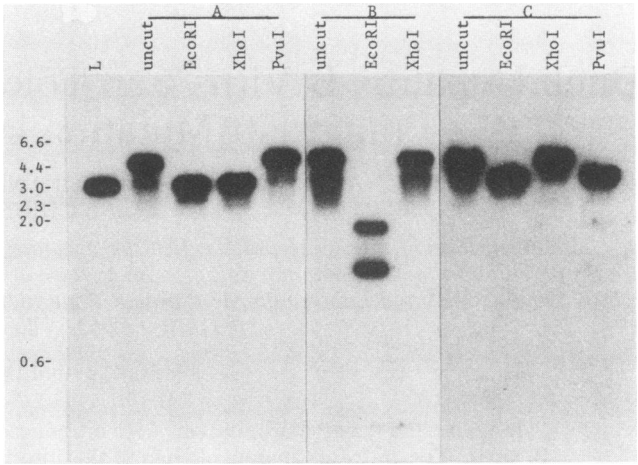

FIG. 3. Restriction enzyme analysis of viral DNA from transfected duck sera. L, Cloned DHBV DNA linearized with *Eco*RI; A, wild-type DHBV DNA; B, in-frame mutant viral DNA (IF-1); C, frameshift mutant viral DNA (FS-17). Molecular sizes in kilobases are indicated at the left.

To investigate whether the progeny viruses still maintain the introduced mutation, the *Eco*RI, *Xho*I, and *Pvu*I restriction patterns of viral DNA were analyzed from transfected duck sera (Fig. 3) and livers (data not shown). Viral DNA from all four ducks transfected with the in-frame mutant DNA contained two *Eco*RI sites and no *Xho*I site, suggesting the maintenance of the in-frame insertion. However, of the seven ducks which received frameshift DNA, only two (ducks 9 and 17) had restriction patterns consistent with those of the transfecting mutant clone (viral DNA from duck 9 was only partially digested with *Pvu*I); the others (ducks 6, 8, 11, 12, and 20) had restriction patterns identical to those of nonmutant prototype DNA, suggesting a possible reversion mutation in these cases.

To directly check for possible changes in the mutation site or adjacent region, viral DNA was cloned from the sera of several representative ducks into the pUC-18 vector. The fragments encompassing the pre-S region (the 1,717-base-pair [bp] *Xba*I-*Xba*I fragment for the in-frame clone and the 1,358-bp *Eco*RI-*Xba*I fragment for the frameshift clone) were subcloned into M13mp18. DNA sequencing (17) demonstrated the maintenance of the introduced 12 nt and no compensatory mutation for an in-frame mutant viral clone (IF-1) (Fig. 4 and Table 1). A frameshift clone with reverted restriction pattern (FS-8) had indeed lost the introduced 4 nt and had therefore reverted to wild type. In the frameshift clone FS-17, the inserted 4 nt were maintained but a 10-nt deletion was found 21 nt downstream of the original mutation site (Fig. 4). For duck 9, two different clones, FS-9a and FS-9b, were obtained. The DNA sequence of FS-9a in the pre-S region was identical to that of FS-17, while in FS-9b, a 10-nt deletion occurred immediately downstream of the 4-nt insertion (Fig. 4). In both FS-17/FS-9a and FS-9b, the reading frame of the pre-S region was restored through deletion of a decanucleotide, but because of the local frameshifting and deletion, four to nine consecutive amino acid codons were changed. All the sequenced mutant clones were subsequently confirmed to be infectious when retransfected into ducklings. To our knowledge, this is the first time that infectious DHBV mutants with altered pre-S regions have been obtained.

To investigate the possible effect of the mutations on viral pre-S protein expression, Western immunoblotting (5, 6) was done by using anti-pre-S monoclonal antibody produced in our laboratory (V. Lambert, D. Fernholz, R. Sprengel, I. Fourel, G. Deléage, G. Wildner, C. Peypet, C. Trépo, L. Cova, and H. Will, submitted for publication). We observed in nonmutant viremic sera one major pre-S protein band of 35 kilodaltons (kDa) and two minor bands of 36 and 28 kDa (Fig. 5); these findings were similar to those of other investigators (14, 19, 24). Since the DHBV pre-S region contains six in-frame ATG codons, these pre-S protein bands may be expressed from different initiation sites. The in-frame mutants exhibited similar but slightly faster migration protein

TABLE 1. Transfection efficiency and DNA sequencing profile of in-frame and frameshift mutant DNAs

DNA clones for transfection	Transfection efficiency (%)	Clones analyzed by DNA sequencing		
		Duck no.	Clone	Sequence modified
In-frame clone: insertion of 12 nt at 1,212 bp	80	1	IF-1	12-nt insertion at 1,212 bp
		17	FS-17	4-nt insertion at 1,212 bp, 10-nt deletion from 1,232 to 1,241 bp
Frameshift clone: insertion of 4 nt at 1,212 bp	28	9	FS-9a	4-nt insertion at 1,212 bp, 10-nt deletion from 1,232 to 1,241 bp
		9	FS-9b	4-nt insertion at 1,212 bp, 10-nt deletion from 1,215 to 1,224 bp
		8	FS-8	Reversion to wild type

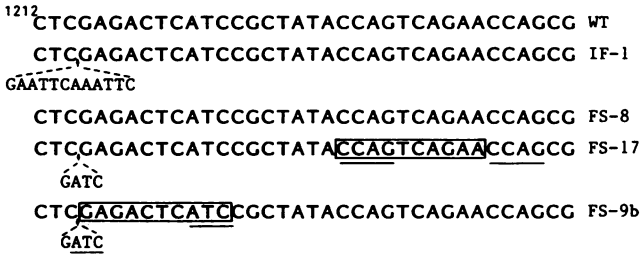

FIG. 4. Sequence comparison of the pre-S regions in wild-type DHBV DNA (WT) and in-frame (IF-1) and frameshift (FS-8, FS-17, and FS-9b) mutant viral DNAs. A portion of the sequence spanning the mutated region is shown in the figure. The inserted nucleotides are indicated by the arrows. The positions of deleted nucleotides are boxed, from 1232 to 1241 in FS-17 and 1215 to 1224 in FS-9b. The repetitive sequences involved in producing deletions are underlined.

bands (Fig. 5), while in partial frameshift mutants (FS-9 and FS-17) the pre-S protein patterns were different from those of the wild type (two major bands of 34 and 35 kDa in FS-9 and one band of 34 kDa in FS-17). Whether the altered protein profiles are due to changes in mobility induced by altered amino acid sequence (3) or reflect the alterations in transcription or translation of the pre-S region is currently under investigation.

Our computer analysis (4, 13) suggested the absence of important potential antigenic sites and a rather low content of regular secondary structures (helices, sheets) in the distal pre-S region of DHBV (data not shown). These secondary structural features were conserved in the FS-17/FS-9a and FS-9b mutants and only slightly changed in the in-frame mutant (data not shown). However, the substitution of even one amino acid, although not changing the secondary structure prediction, may change the local tertiary structure and affect the viral biological properties (15, 23).

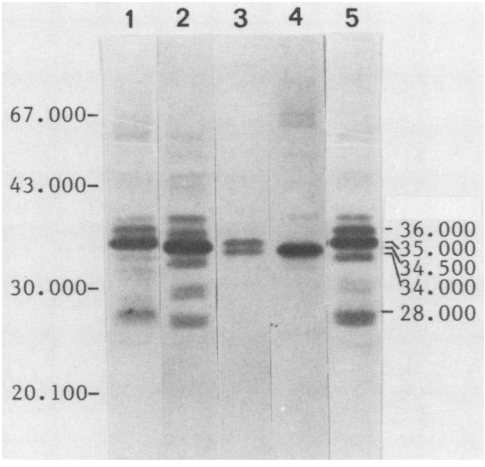

FIG. 5. Western immunoblotting analysis of the pre-S protein profiles of the DHBV mutants by using monoclonal anti-pre-S antibody. Virus concentrated from viremic sera was heat denatured, and proteins were separated on sodium dodecyl sulfate–12.5% polyacrylamide gels (6). The proteins were electrotransferred onto a nitrocellulose filter (5), incubated with anti-pre-S mouse monoclonal antibody, and then revealed by horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (TAGO, Inc.) and developed with 3,3′-diaminobenzidine (Sigma Chemical Co.). Lane 1, Wild-type DHBV; lane 2, in-frame mutant (IF-1); lanes 3 to 5, frameshifted mutants FS-9 (3), FS-17 (4), and FS-8 (5). Molecular masses in daltons are indicated at the left. The positions of the main pre-S protein bands in daltons are indicated at the right.

In conclusion, our results suggest that at least part of the late pre-S region is not critical for viral infectivity, since it can undergo insertional, deletional, and partial frameshift mutations spanning nt 1212 to 1241 without loss of replication capacity. Whether specific residues in this area are required for local topology, as suggested by the differences observed in the pre-S protein patterns and more transient viremia in some mutants, is currently under investigation.

In addition, as the DNA sequence in the pre-S region codes for both the pre-S protein and the DNA polymerase, our results also suggest that the amino acid sequence of the DNA polymerase within the late pre-S region is not crucial. This is supported by a recent mutational study of the P ORF, which showed that the pre-S portion of the P gene is a nonessential spacer (1).

The frameshift mutation appears to be detrimental to DHBV replication, as evidenced by the reverse and compensatory mutations. This may be due to the frameshift of the S moiety in the pre-S/S protein preventing anchorage of the protein on the surface of the virion, to the +1 frameshift of the DNA polymerase gene leading to premature termination of the translation of the enzyme at position 1255, or to both circumstances. The reverse mutants are not likely to have been generated by replication of a minute amount of contaminating wild-type DHBV, as the mutant DNA was prepared by six rounds of colony purification and digested with *Xho*I to prevent the replication of the wild-type DHBV. Of particular interest is the compensatory deletion of a decanucleotide at different sites in the two mutant virus stocks (FS-17/FS-9a and FS-9b). Examination of the adjacent nucleotides revealed short repeated flanking sequences in both cases (Fig. 4). Deletion of nucleotides through repetitive sequence has been documented in retrovirus replication (12, 16). Our results suggest that a similar mechanism of DNA repair may be operative for hepadnavirus.

We thank Chantal Jacquet for excellent technical assistance and Brigitte Roux for typing the manuscript.

LITERATURE CITED

1. **Bartenschlager, R., and H. Schaller.** 1988. The amino-terminal domain of the hepadnaviral P-gene encodes the terminal protein (genome-linked protein) believed to prime reverse transcription. EMBO J. **7**:4185–4192.
2. **Cova, L., V. Lambert, A. Chevallier, O. Hantz, I. Fourel, C. Jacquet, C. Pichoud, J. Boulay, B. Chomel, L. Vitviski, and C. Trépo.** 1986. Evidence for the presence of duck hepatitis B virus in wild migrating ducks. J. Gen. Virol. **67**:537–547.
3. **De Jong, W. W., A. Zweers, and L. H. Cohen.** 1978. Influence of single amino acid substitution on electrophoretic mobility of sodium dodecyl sulfate-protein complexes. Biochem. Biophys. Res. Commun. **82**:532–539.
4. **Deléage, G., F. F. Clerc, B. Roux, and D. C. Gautheron.** 1988. Antheprot: a package for protein sequence analysis using a microcomputer. Comput. Appl. Biosci. **4**:351–356.
5. **Kyhse-Andersen, J.** 1984. Electroblotting of multiple gels using a simple apparatus without buffer tank for rapid transfer protein from polyacrylamide to nitrocellulose. J. Biochem. Biophys. Methods **10**:203–209.
6. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
7. **Mandart, E., A. Key, and F. Galibert.** 1984. Nucleotide sequence of a cloned duck hepatitis B virus genome: comparison with woodchuck and human hepatitis B virus sequences. J. Virol. **49**:782–792.
8. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular

cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

9. **Milich, D. R., G. B. Thornton, A. R. Neurath, S. B. Kent, M. L. Michel, P. Tiollais, and F. V. Chisari.** 1985. Enhanced immunogenicity of the pre-S region of hepatitis B surface antigen. Science **228**:1195–1198.
10. **Neurath, A. R., S. Kent, K. Parker, A. Prince, N. Strick, B. Brotman, and P. Sproul.** 1986. Antibodies to a synthetic peptide from the pre-S 120-145 region of the HBV envelope are virus neutralizing. Vaccine **4**:35–37.
11. **Neurath, A. R., S. B. H. Kent, N. Strick, and K. Parker.** 1986. Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus. Cell **46**:429–436.
12. **Omer, C. A., K. Pogue-Geile, R. Guntaka, K. A. Staskus, and A. J. Faras.** 1983. Involvement of directly repeated sequences in the generation of deletions of the avian sarcoma virus *src* gene. J. Virol. **47**:380–382.
13. **Parker, J. M. R., D. Guo, and R. S. Hodges.** 1986. New hydrophilicity scale derived from high-performance liquid chromatography peptide retention data: correlation of predicted surface residues with antigenicity and X-ray-derived accessible sites. Biochemistry **25**:5425–5432.
14. **Pugh, J. C., J. J. Sninsky, J. W. Summers, and E. Schaeffer.** 1987. Characterization of a pre-S polypeptide on the surfaces of infectious avian hepadnavirus particles. J. Virol. **61**:1384–1390.
15. **Reitz, M. S., Jr., C. Wilson, C. Naugle, R. C. Gallo, and M. Robert-Guroff.** 1988. Generation of a neutralization-resistant variant of HIV-1 is due to selection for a point mutation in the envelope gene. Cell **54**:57–63.
16. **Rhode, B. W., M. Emerman, and H. W. Temin.** 1987. Instability of large direct repeats in retrovirus vectors. J. Virol. **61**: 925–927.
17. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
18. **Schaeffer, E., and J. J. Sninsky.** 1984. Predicted secondary structure similarity in the absence of primary amino acid sequence homology: hepatitis B virus open reading frames. Proc. Natl. Acad. Sci. USA **81**:2902–2906.
19. **Schlicht, H. J., C. Kuhn, B. Guhr, R. J. Mattaliano, and H. Schaller.** 1987. Biochemical and immunological characterization of the duck hepatitis B virus envelope proteins. J. Virol. **61**:2280–2285.
20. **Sprengel, R., E. F. Kaleta, and H. Will.** 1988. Isolation and characterization of a hepatitis B virus endemic in herons. J. Virol. **62**:3832–3839.
21. **Sprengel, R., C. Kuhn, C. Manso, and H. Will.** 1984. Cloned duck hepatitis B virus DNA is infectious in Pekin ducks. J. Virol. **52**:932–937.
22. **Tiollais, P., C. Pourcel, and A. Dejean.** 1985. The hepatitis B virus. Nature (London) **317**:489–495.
23. **Willey, R. L., D. H. Smith, L. A. Lasky, T. S. Theodore, P. L. Earl, B. Moss, D. J. Capon, and M. A. Martin.** 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J. Virol. **62**:139–147.
24. **Yokosuka, O., M. Omata, and Y. Ito.** 1988. Expression of pre-S1, pre-S2 and C proteins in duck hepatitis B virus infection. Virology **167**:82–86.