JOURNAL OF VIROLOGY, Mar. 1993, p. 1218–1226
0022-538X/93/031218-09$02.00/0
Copyright © 1993, American Society for Microbiology
Vol. 67, No. 3

The Woodchuck Hepatitis Virus X Gene Is Important for Establishment of Virus Infection in Woodchucks

HONG-SHU CHEN,[1,2] SHUICHI KANEKO,[1]† ROSINA GIRONES,[1]‡ ROBERT W. ANDERSON,[3] WILLIAM E. HORNBUCKLE,[4] BUD C. TENNANT,[4] PAUL J. COTE,[5] JOHN L. GERIN,[5] ROBERT H. PURCELL,[1] AND ROGER H. MILLER[1]*

Hepatitis Viruses Section, Laboratory of Infectious Diseases,[1] and Laboratory of Viral Diseases,[3] National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892; Department of Immunology and Infectious Diseases, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Maryland 21205[2]; College of Veterinary Medicine, Cornell University, Ithaca, New York 14853[4]; and Division of Molecular Virology and Immunology, Georgetown University, Rockville, Maryland 20852[5]

Received 27 July 1992/Accepted 7 December 1992

All mammalian hepadnaviruses possess a gene, termed X, that encodes a protein capable of transactivating virus gene expression. The X gene overlaps the polymerase and precore genes as well as two newly identified open reading frames (ORFs) termed ORF5 and ORF6. In this investigation, we examined whether ORF5, ORF6, and the X gene were important for the replication of woodchuck hepatitis virus (WHV) in susceptible woodchucks. First, we investigated whether proteins were produced from ORF5 and ORF6 by in vitro translation of appropriate viral transcripts, searched for antibodies against the putative proteins in the sera of animals infected with wild-type virus, and looked for an antisense WHV transcript, necessary for expression of a protein from ORF6, in the livers of acutely or chronically infected woodchucks. All such experiments yielded negative results. Next, we used oligonucleotide-directed mutagenesis to introduce termination codons into ORF5 and ORF6 at two locations within each ORF. Adult woodchucks in groups of three were transfected with one of the four mutant genomes. All of these woodchucks developed WHV infections that were indistinguishable from those of animals transfected with the wild-type WHV recombinant. Polymerase chain reaction amplification and direct DNA sequencing confirmed that reversion of the mutants to a wild-type genotype did not occur. Taken together, these data indicate that ORF5 and ORF6 are not essential for virus replication and are unlikely to represent authentic genes. Finally, we generated five WHV X-gene mutants that either removed the initiation codon for protein synthesis or truncated the carboxyl terminus of the protein by 3, 16, 31, or 52 amino acids. Groups of three adult woodchucks were transfected with one of the five X-gene mutants. Only the mutant that possessed an X gene lacking 3 amino acids from the carboxyl terminus was capable of replication within the 6-month time frame of the experiment. In contrast, all seven woodchucks transfected with wild-type WHV DNA developed markers consistent with viral infection. Thus, it is likely ($P < 0.01$) that the WHV X gene is important for virus replication in the natural host.

The family *Hepadnaviridae* (12) contains at least six members that are classified as avian (duck hepatitis B virus [HBV] [23] and heron HBV [45]) or mammalian (HBV [4], woodchuck hepatitis virus [WHV] [47], ground squirrel hepatitis virus [22], and tree squirrel hepatitis virus [8]) hepadnaviruses. The main difference in genomic organization between these two virus groups is that the mammalian hepadnaviruses encode an additional gene, the X gene (9).

The genome of mammalian hepadnaviruses is a partially double-stranded circle that is 3.2 to 3.3 kb in size. The genome is organized in a highly complex and economical way such that the four known genes (surface, core, polymerase, and X) overlap extensively. The consequence of this fact is that ≈50% of the nucleotides are used to encode more than one gene. In addition, all of the *cis*-acting control elements are located within the protein-coding sequences (29). While the core, polymerase, and surface proteins are essential in the virus life cycle, the role of the X protein is not clear (18). The HBV X protein has been shown to possess transcriptional *trans*-activating activity in in vitro assays (2, 5, 19, 39, 41, 43, 44, 51–53, 58), to share similarity to the "Kunitz domain" of serine protease inhibitors (50), and to exhibit a serine/threonine protein kinase activity (60). The HBV X protein has been detected in liver tissue from patients with chronic hepatitis B, cirrhosis, and hepatoma (55, 56). Although the X protein does not appear to be necessary for virus replication in cultured cells (3, 16, 61), it is not yet known whether this gene product is essential for replication in vivo.

Infection of woodchucks with WHV has proven to be an excellent model system for the study of HBV infection of humans (10). We used oligonucleotide-directed mutagenesis of the X gene of an infectious WHV recombinant (11) to determine whether the product of this gene was important for virus replication in the natural host. In preparation for mutagenesis of the WHV X gene, we performed a computer analysis of the published genome sequences of hepadnaviruses to define conserved nucleotide sequences in order to avoid mutating known or unidentified *cis*-acting control sequences and to identify open reading frames (ORFs) that could represent unidentified genes. We found that the X-gene region of the hepadnaviral genome, as well as the region of the genome of certain retroviruses that encodes proteins involved in stimulating viral transcription, is char-

* Corresponding author.
† Present address: First Department of Internal Medicine, Kanazawa University, Kanazawa, Japan.
‡ Present address: Department of Microbiology, University of Barcelona, Barcelona, Spain.

acterized by the absence of termination codons in the six possible translation frames of the double-stranded DNA replicative intermediates (26, 27). Thus, we identified two new ORFs, designated ORF5 and ORF6, in the genomes of all hepadnaviruses examined (14, 25, 29, 48).

Further analyses showed that ORF5 ranges from 70 to over 100 codons in size (103 amino acids in the genome of a WHV recombinant, WHV8 [11]; map positions 1493 to 1801) and overlaps with the polymerase and X genes (Fig. 1). ORF5 lacks an AUG initiation codon, and a protein encoded by this ORF would have to be expressed by a mechanism involving frameshifting (e.g., from the X gene), or from a spliced mRNA (i.e., with an AUG codon fused in phase with ORF5), or by using an unconventional initiation codon (e.g., ACG). In fact, there is an ACG codon near the 5′ end of ORF5 in all hepadnaviral isolates examined. Thus, the protein encoded by ORF5 could be translated from any of the previously identified virus mRNA transcripts or from a spliced version of the known transcripts.

On the other hand, ORF6 is located on the viral DNA strand that is complementary to the one that encodes the known virus proteins. Thus, ORF6 could not be expressed from any of the previously identified mRNA transcripts but would be translated from an antisense mRNA molecule. ORF6 is ≈200 amino acids in length in hepadnaviral genomes (179 amino acids in the WHV8 genome [11]; map positions 1719 to 1183) and also overlaps the polymerase and X genes of the mammalian hepadnaviruses (Fig. 1). There is an AUG initiation codon near the amino terminus of the ORF in HBV genomes but not in WHV genomes. It is not known whether these new ORFs are authentic genes, represent vestigial gene sequences conserved through evolution, or have no significance. Since both ORF5 and ORF6 overlap the X gene, it seemed prudent to investigate the importance of these ORFs in viral replication before beginning analysis of the X gene.

In this study, we determined whether proteins were produced from ORF5 and ORF6 by assay of in vitro translation, searched for antibodies against the putative proteins in the sera of animals infected with wild-type virus, and looked for an antisense WHV transcript in the livers of infected woodchucks. Next, we performed site-directed mutagenesis of ORF5 and ORF6 in an infectious cDNA clone of WHV and evaluated them in woodchuck transfection experiments. Finally, we constructed five WHV X-gene mutants that either removed the initiation codon for protein synthesis or truncated the carboxyl terminus of the protein by 3, 16, 31, or 52 amino acids, and evaluated their ability to replicate following transfection in woodchucks.

MATERIALS AND METHODS

Construction of plasmids and in vitro transcription. WHV8 DNA (11) was subcloned into vector pTZ18, pTZ19, or pGEM (Pharmacia, Piscataway, N.J.) by standard methods (21). Specifically, the *Acc*I (position 953)-*Sph*I (position 1772) region was cloned into vector pTZ18 [recombinant pTZ18(A-S)] or pTZ19 [recombinant pTZ19(A-S)] to generate strand-specific probes. The complete WHV genome as well as the *Nci*I (position 1367)-*Hin*dIII (position 2190) region was cloned into vector pGEM. RNA transcripts were prepared by using T7 RNA polymerase according to the instructions of the supplier in the presence or absence of [^{32}P]CTP. The specific activities of radiolabeled transcripts were 1×10^9 to 2×10^9 cpm/μg of DNA. The transcript synthesized from recombinant pTZ18(A-S) is complementary to negative-strand transcripts of the WHV genome, while the transcript from recombinant pTZ19(A-S) is complementary to positive-strand WHV transcripts.

Northern (RNA) blot hybridization. Whole-cell RNA was isolated following homogenization of the liver tissue in 4 volumes of 4 M guanidine isothiocyanate and centrifugation through a 5.7 M cesium chloride cushion (21). Poly(A)$^+$ RNA in loading buffer (20 mM Tris hydrochloride [pH 7.4], 1 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 0.5 M NaCl) was selected by two rounds of oligo(dT)-cellulose chromatography (type 7; Pharmacia). Poly(A)$^-$ RNA was obtained by extensive washing with loading buffer. Columns were then rinsed with washing buffer (20 mM Tris hydrochloride [pH 7.4], 1 mM EDTA, 0.1% SDS, 0.1 M NaCl), and poly(A)$^+$ RNA was eluted with a solution containing 10 mM Tris HCl (pH 7.4), 1 mM EDTA, and 0.1% SDS (21). Liver cells were fractionated as previously described (32). RNA was purified from the nuclear and cytoplasmic fractions as described above. Thirty micrograms of whole-cell or nuclear RNA or 4 μg of poly(A)$^+$ RNA was fractionated by electrophoresis through a 1.5% agarose gel. Northern blot hybridization was performed as previously described (14).

The woodchucks used for detection of WHV transcripts were housed at the animal facility of the College of Veterinary Medicine, Cornell University (Ithaca, N.Y.). One woodchuck (WC809) had no evidence of a past or current WHV infection and served as a negative control. All woodchucks used for experimental infection were the offspring of WHV-negative mothers and were part of a study of the natural history of WHV infection of woodchucks (17). A total of 43 woodchucks (3 to 7 days old) were each inoculated subcutaneously with 100 μl of WHV8-infected woodchuck serum containing $\approx 5 \times 10^6$ 50% woodchuck infectious doses and kept in isolation. Animals were sacrificed at 4, 8, 14, 18, 28, 42, and 65 weeks after birth. The animals used in our investigation were sacrificed as follows: three woodchucks sacrificed at week 4 (WC1477, WC2020, and WC2027), three woodchucks sacrificed at week 8 (WC1638, WC1646, and WC1648), seven woodchucks sacrificed at week 28 (WC1635, WC1636, WC1651, WC1655, WC2016, WC2022, and WC2025), five woodchucks sacrificed at week 42 (WC1643, WC1644, WC1664, WC2023, and WC2024), and three woodchucks sacrificed at week 65 (WC1290, WC1408, and WC1613). The woodchucks sacrificed 4 to 8 weeks postinoculation were negative for both WHV surface antigen (WHsAg) and antibodies against the WHV surface (anti-WHs); however, the woodchucks sacrificed more than 8 weeks postinoculation were positive for WHV DNA (17). The older woodchucks were either positive for WHsAg (WC1408, WC1643, WC1651, WC2016, WC2022, WC2024, and WC2025) or anti-WHs (WC1290, WC1613, WC1635, WC1636, WC1644, WC1655, WC1664, and WC2023).

Immunological assays. Synthetic peptides were synthesized on a MilliGen 9050 peptide synthesizer (MilliGen/Biosearch, Burlington, Mass.) by using the manufacturer's reagents and recommended synthesis methods. Peptides were cleaved from the resin, precipitated, and extracted several times with anhydrous ether and fractionated by gel filtration and/or high-performance liquid chromatography (HPLC). Amino acid composition analysis, amino acid sequence analysis, and reverse-phase HPLC were performed to confirm peptide sequence and purity. The synthetic peptides used in this study were peptide 604 (CHTGSNS MIQRH) specific for ORF6, peptide 621 (CKSSTWHAK) specific for ORF5, and peptide 665 (CRHKCMRLL) specific for the X protein, deduced from the carboxyl termini of the

respective amino acid sequences, and peptide 92 (LQPT TGTTVNCRQCTISAQNMYTPPYC) was deduced from the center of the surface protein. Antisera against peptides 92, 604, 621, and 665 were prepared in rabbits immunized with peptides conjugated to keyhole limpet hemocyanin KLH (Calbiochem, San Diego, Calif.) with the crosslinker *m*-maleimido benzoyl-*N*-hydroxysuccinimide ester (MBS) (Pierce, Rockford, Ill.). The specificities of the antibodies were verified by radioimmunoassay.

Radioimmunoassay was used to search for antibodies against the peptides in infected woodchuck sera. Briefly, peptides (50 μl of 1 mg/ml in 0.1% phosphate-buffered saline [PBS]) were coated onto microtiter plates and incubated with WHV-infected woodchuck serum. Any captured antibodies were quantified by the binding of ^{125}I-labeled rabbit anti-woodchuck immunoglobulin G, with 2×10^5 cpm added to each well. Peptide 92, specific for the WHV surface protein, served as a positive control in the study. The woodchucks used in the analysis were either uninfected animals used as negative controls or experimentally infected animals that developed acute or chronic WHV infections. Serial serum samples were collected twice per month or on a monthly basis. A total of 69 samples were analyzed from uninfected woodchucks: WC463 (18 samples), WC464 (17 samples), WC466 (16 samples), and WC467 (18 samples). A total of 253 samples were analyzed from WHV-infected woodchucks: WC845 (18 samples), WC847 (20 samples), WC848 (19 samples), WC850 (20 samples), WC862 (19 samples), WC1210 (14 samples), WC1211 (17 samples), WC1224 (17 samples), WC1237 (19 samples), WC1238 (17 samples), WC1239 (19 samples), WC1276 (14 samples), WC1277 (13 samples), WC1409 (13 samples), and WC1447 (14 samples).

In vitro translation. Transcripts from the various constructs were translated by using a rabbit reticulocyte lysate system (Promega, Madison, Wis.) in the presence of [^{35}S]methionine according to the instructions of the manufacturer. A 10-μl aliquot of the lysate was incubated at 4°C overnight with 5 μl of the appropriate antiserum (diluted 1:50, 1:250, or 1:1,250) and then treated with protein A-Sepharose (Pharmacia) at 4°C for 30 min. The Sepharose then was pelleted and washed three times with buffer (PBS, 0.1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate). After being heated for 3 min in a boiling water bath, the samples were fractionated by electrophoresis on 15% polyacrylamide gels containing SDS and the radiolabeled proteins were detected by autoradiography.

Oligonucleotide-directed mutagenesis of the WHV genome. The genome of an infectious WHV recombinant, WHV8 (11), was cloned into the *Eco*RI site of the phagemid pBluescript II KS(+) (Stratagene, La Jolla, Calif.). The complete nucleotide sequence of the 3.3-kb genome was determined by the dideoxy-chain termination method of Sanger and coworkers (40) using Sequenase (United States Biochemical, Cleveland, Ohio) and was found to be identical to the parental clone. Single-stranded DNA, generated with helper phage VCS-M13 (Stratagene) served as the template for oligonucleotide-directed mutagenesis (Amersham, Arlington Heights, Ill.) by the previously described method of Nakamaye and Eckstein (35). Briefly, oligonucleotides (30 nucleotides in length) were synthesized in an oligonucleotide synthesizer (Applied Biosystems, Foster City, Calif.) with a single-nucleotide substitution near the center of the molecule. The oligonucleotides were annealed to the single-stranded WHV DNA template and extended by Klenow polymerase in the presence of T4 DNA ligase to generate a mutant heteroduplex. Selective removal of the nonmutant strand was made possible by protection of the mutant strand from digestion by restriction endonuclease *Nci*I and exonuclease III by the incorporation of thionucleotides into the mutant strand during in vitro synthesis. Double-stranded mutant homoduplex molecules were generated by treatment with DNA polymerase I, followed by ligation by T4 DNA ligase, and were used to transform competent *Escherichia coli* cells. The presence of the mutations was confirmed by nucleotide sequencing of the mutated genome regions.

Transfection of adult woodchucks with recombinant WHV DNA. WHV recombinants with mutated sequences were propagated in *E. coli*, the DNA was purified by standard methods, and the WHV DNA insert was released from the vector sequence by digestion with *Eco*RI (21). WHV DNA was isolated by agarose gel electrophoresis and electroelution. WHV DNA was treated with T4 DNA ligase at DNA concentrations (5 to 10 μg of DNA per ml) that are optimal for the production of monomeric, circular genomes (11, 28). As a precaution against the effect of lethal mutations arising outside the mutated region of the genome in a given recombinant during the process of cloning and also to eliminate the necessity of sequencing the complete genome of each mutant, we prepared in parallel two individual recombinants bearing an identical mutation. The mutant pairs were mixed in equal amounts at the final resuspension step for inoculation of the animals.

Recombinant DNA was resuspended in PBS and injected into six different locations of the surgically exposed woodchuck livers. Three animals were transfected with each mutant, and eight animals were inoculated with wild-type WHV8 DNA as a positive control. Each woodchuck received a dose of 50 μg of purified monomeric WHV DNA in a volume of 0.5 ml of PBS. Starting 1 month after transfection, serum samples were collected biweekly for a total of 4 months, and samples were collected monthly thereafter. The woodchucks transfected with recombinant WHV were as follows: WC173, WC180, WC181, WC272, WC275, WC282, WC2550, and WC2746 (wild-type WHV8 DNA); WC2188, WC2531, and WC2735 (ORF5 mutant 1 DNA); WC2323, WC2551, and WC2571 (ORF5 mutant 2 DNA); WC885, WC2302, and WC2738 (ORF6 mutant 1 DNA); WC876, WC2154, and WC2561 (ORF6 mutant 2 DNA); WC892, WC2406, and WC2745 (X mutant 1); WC2718, WC2734, and WC2832 (X mutant 2); WC2435, WC2706, and WC2918 (X mutant 3); WC2556, WC2712, and WC2742 (X mutant 4); and WC2831, WC2865, and WC2873 (X mutant 5). All of the experimental woodchucks were the offspring of WHV-free parents that were born and raised in laboratory animal facilities. After transfection with recombinant WHV DNA, all woodchucks were housed in isolation to prevent horizontal spread of infection.

Infection of neonatal woodchucks with serum-derived WHV. Neonatal woodchucks born to WHV-free female woodchucks were used to examine the infectivity and pathogenesis of infection of progeny WHV particles from recombinant WHV DNA-transfected adult woodchucks. The serum sample from WC2873 (transfected with X mutant 5) was first WHsAg positive 6 weeks posttransfection, and the serum sample collected at week 10 was used to infect neonates. A total of 100 μl of serum, containing approximately 2.7×10^8 genomes (determined by slot blot hybridization), was injected subcutaneously into each of six neonatal woodchucks (WC1879 to WC1884) on the sixth day postpartum as previously described (17, 28). Starting 2

months after injection, serum samples were collected monthly for serological monitoring of WHV infection.

Serological assays for markers of WHV infection. Serum samples collected serially were tested for the presence of WHsAg as well as anti-WHs and antibodies against WHV core (anti-WHc) proteins using solid-phase radioimmunoassay or enzyme-linked immunosorbent assays (6, 36, 59).

Amplification of WHV DNA from woodchuck serum by PCR. WHV DNA from all WHsAg-positive serum samples was amplified by two rounds of polymerase chain reaction (PCR) assay as previously described (13), using nested primer pairs bracketing the mutated region of the genome. The PCR products were fractionated by 2% agarose gel electrophoresis and isolated by electroelution. The DNA fragments were sequenced directly (i.e., without cloning) by a modified dideoxy-chain termination method in the presence of dimethyl sulfoxide (57).

Slot blot hybridization of WHV DNA from serum samples. WHV DNA was purified from 50-μl portions of WHsAg-positive serum samples by digestion with proteinase K, extraction with phenol and chloroform, and precipitation with alcohol. DNA was denatured by NaOH and transferred to a nitrocellulose membrane for detection by hybridization (30) with a ^{32}P-radiolabeled, WHV DNA-specific probe (7). WHV DNA was detected by autoradiography.

RESULTS

Prior to experiments on the mutagenesis of the WHV X gene, a computer analysis of all published hepadnavirus sequences was performed to map invariant regions that could represent crucial *cis*-acting signal sequences embedded within the coding domain of the X gene and to look for unidentified ORFs. One outcome of this analysis was the identification of two ORFs that were present in all hepadnavirus genomes examined and overlapped the X-gene sequence of the mammalian hepadnaviruses. We performed experiments (see below) to test whether ORF5 and ORF6 were authentic genes and were utilized in virus replication before performing site-directed mutagenesis on the WHV X gene to determine whether this gene was important for viral replication in the natural host.

Immunoassay to detect antibodies against ORF5 or ORF6 proteins in acutely or chronically infected woodchucks. In this study, we used a radioimmunoassay incorporating synthetic peptides specific for the amino acid sequence of ORF5 and ORF6 to detect circulating antibodies in woodchucks with acute or chronic WHV infection. Peptides were synthesized so that they would correspond to the predicted carboxyl termini of the putative ORF5 and ORF6 proteins, and rabbit antisera were produced (see Materials and Methods). A peptide specific for the WHsAg (peptide 92) was used as a positive control in the analysis. A total of 253 serum samples from 15 different woodchucks were tested in the assay. Comparable amounts of rabbit anti-woodchuck immunoglobulin G were bound in the assays using serum samples from the WHV-infected woodchucks and in 69 serum samples from four uninfected woodchucks. In contrast, antibodies against peptide 92 were detected in woodchucks positive for anti-WHs. Thus, we did not detect antibodies against the ORF5 and ORF6 proteins in sera during the course of WHV infection.

In vitro translation of WHV RNA. Although neither ORF5 nor ORF6 possesses an in-phase AUG codon at the amino terminus, it is possible that proteins could be translated from these ORFs by use of an unconventional initiation codon or, in the case of ORF5, by a frameshifting mechanism utilizing the X ORF. Therefore, we cloned the relevant regions of the WHV genome into RNA expression vectors and produced viral transcripts for translation in a rabbit reticulocyte lysate assay (see Materials and Methods). Although the WHV X protein was readily detected by using antibody against a synthetic peptide specific for the X protein (see Materials and Methods), proteins translated from ORF5 or ORF6-specific transcripts could not be detected by using specific antibodies (data not shown).

Examination of WHV-infected woodchuck livers for evidence of an antisense viral transcript. In WHV infection of hepatocytes, three virus-specific transcripts are produced. Two transcripts possess poly(A) tails and are 2.3 and 3.6 kb in size (14, 34) while one transcript, specific for the X-gene region of the genome, lacks a poly(A) tail, is ≈0.7 kb in size, and is primarily found in the nuclei of infected cells (14). The X-gene-specific transcript is ≈1% of total virus RNA. In this study, we used Northern blot hybridization with strand-specific probes to determine whether an antisense transcript was produced in the livers of woodchucks during the course of experimental WHV infection. We found that the 2.3- and 3.6-kb transcripts were readily detected with a probe specific for positive polarity transcripts in total RNA or poly(A)-selected RNA. In addition, the 0.7-kb transcript was detected in the nuclear fraction of infected woodchuck hepatocytes as described in our previous study (14). However, we were unsuccessful in detecting an antisense transcript with a probe specific for negative polarity transcripts in total RNA, poly(A)-selected RNA, or nuclear RNA of 21 woodchucks with either acute or chronic WHV infections (see Materials and Methods). Although it is possible that an antisense transcript is present at very low levels in infected cells (<0.1% of virus-specific RNA), we believe that it is unlikely that such a transcript is produced during the course of WHV infection. Although we cannot rule out the possibility that ORF5 or ORF6 is expressed at low levels, the data from these studies suggest that neither of the ORFs encodes a protein.

Oligonucleotide-directed mutagenesis of WHV ORF5 and ORF6. We used oligonucleotide-directed mutagenesis to introduce point mutations in ORF5 or ORF6 to determine whether these two ORFs are essential for viral replication in vivo. A total of four WHV mutants were constructed, with each containing a single point mutation that changed a codon specifying an amino acid into a termination codon truncating either ORF5 or ORF6 (Fig. 1). In ORF5 mutants 1 and 2, the ORF was truncated by 52 (50%) and 77 (75%) amino acids, respectively. In ORF6 mutants 1 and 2, the ORF was truncated by 102 (58%) and 69 (39%) amino acids, respectively. We avoided making changes in any of the *cis*-acting control elements in this region and in making any drastic changes in the overlapping genes (Table 1). Next, the WHV mutants were tested for their ability to replicate in woodchucks. Fourteen adult woodchucks, three animals for each of the four mutant viruses and two animals for the wild-type virus positive control, were used in the in vivo transfection study. During the study, two woodchucks died of causes unrelated to WHV transfection (WC2746 transfected with wild-type WHV DNA and WC2735 transfected with ORF5 1 DNA). All of the remaining woodchucks developed serological evidence of WHV replication as early as 8 weeks posttransfection. The serological profile of anti-WHc, WHsAg, and anti-WHs from serial serum samples was similar among the transfected woodchucks. In addition, in each group a similar proportion of animals (at least one of three) became

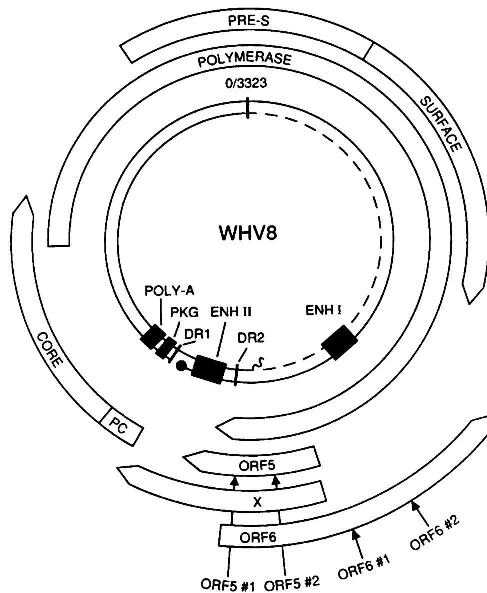

FIG. 1. Genome structure of WHV. The unique *Eco*RI recognition site is designated position 0 and 3323 (9). The presurface (PRE-S), surface, precore (PC), core, polymerase, and X genes are shown. *cis*-Acting regulatory elements highlighted are enhancer I (ENH I), enhancer II (ENH II), direct repeats 1 and 2 (DR1 and DR2), the RNA packaging signal (PKG), and the poly(A) addition signal (POLY-A). ORF5 and ORF6 are shown, with the locations of premature termination mutations indicated by arrows.

chronic carriers of WHsAg (Fig. 2). These results suggest that ORF5 and ORF6 are not essential for viral replication in the natural host.

Serum samples collected during the peak level of surface antigenemia were selected from each group of woodchucks for sequence analysis of WHV DNA (Fig. 2). WHV DNA was isolated from the serum, amplified by PCR, and the DNA was sequenced directly. The sequence of the ORF5 or ORF6 regions of the progeny WHV DNA genomes from the

TABLE 1. Summary of ORF5 and ORF6 mutants

Mutant virus (map position of substitution[a])	Mutation	Changes in overlapping gene(s) or ORF(s)
ORF5		
1 (WHV 1646T)	AGA→TGA	ORF5, truncated by 52 amino acids (50%); polymerase, Gln[b]→Leu; ORF6, Leu→Gln
2 (WHV 1572G)	TCA→TGA	ORF5, truncated by 77 amino acids (75%); X, Gln[c]→Glu; ORF6, Glu→Gln
ORF6		
1 (WHV 1491A)	CAG→TAG	ORF6, truncated by 102 amino acids (58%)
2 (WHV 1389A)	GAG→TAG	ORF6, truncated by 69 amino acids (39%)

[a] Map position on the plus strand of the single-nucleotide substitution, with the unique *Eco*RI site as position 0 or 3323.
[b] This amino acid is highly variable in mammalian hepadnaviruses.
[c] All HBV genomes encode a Glu at this position.

sera of the transfected animals was found to be identical to that of the input, parental WHV DNA. Thus, there was no evidence for reversion to a wild-type genotype.

One important indicator of viral replication is the titer of WHV DNA in sera. Therefore, we quantified the level of WHV DNA by slot blot hybridization. WHV DNA was extracted from the serum samples collected during the peak of surface antigenemia from selected chronic carriers from each group (WC885, WC2154, WC2531, WC2550, and WC2551). Slot blot hybridization of these WHV DNA samples showed that the level of viral DNA in the sera of woodchucks transfected with mutant WHV DNA (1 to 5 ng/ml) was comparable to that in woodchucks transfected with wild-type WHV DNA (1 to 2 ng/ml) in this study (Fig. 3) as well as in previous studies (0.5 to 20 ng/ml). Overall, transfection of woodchucks with WHV mutants containing a premature termination codon in ORF5 or ORF6 resulted in infections that were indistinguishable from those seen in wild-type WHV DNA-transfected animals.

Oligonucleotide-directed mutagenesis of WHV X gene. The second phase of the mutagenesis study was to examine the role of the product of the X gene in virus replication. A series of five X-gene mutants was constructed either to remove the AUG initiation codon (X mutant 1) or to truncate the carboxyl terminus of the X protein by 52 (37%), 31 (22%), 16 (11%), or 3 (2%) amino acids (X mutants 2 to 5) (Table 2). The same precautions taken in designing the ORF5 and ORF6 mutants were followed to avoid any dramatic changes in the overlapping polymerase and precore genes and in *cis*-acting control elements (Fig. 4). Three adult woodchucks were transfected with each of the five X-gene mutants. Measurement of serological markers demonstrated that only X mutant 5 (lacking 3 amino acids at the carboxyl terminus) was capable of replication after transfection into the livers of susceptible animals. The remaining 12 animals transfected with X mutants 1 to 4 were serologically negative for at least 24 weeks (Fig. 5). In our experience, adult woodchucks transfected with infectious WHV DNA typically become positive for serological markers of WHV infection within 16 weeks posttransfection (unpublished data).

WHV DNA was extracted from the sera of WC2873 (transfected with X mutant 5) at the peak of surface antigenemia and was amplified in a nested PCR assay, and the portion of the genome containing the mutation was directly sequenced. The results indicated that the mutated sequence was present in the progeny virus derived from sera and demonstrated that reversion to a wild-type genotype had not occurred. Slot blot hybridization of WHV DNA derived from the serum of the transfected woodchuck revealed that its concentration (1 ng/ml) was within the range seen in a wild-type WHV infection. The same serum sample was used to inoculate six neonatal woodchucks. All six neonates showed serological evidence of WHV infection 12 weeks after inoculation, and one became chronically infected. Taken together, these data suggest that the WHV X gene is important for the establishment of virus infection in the natural host.

DISCUSSION

In this study, we have demonstrated that neither ORF5 nor ORF6 is essential for WHV replication in the natural host. In addition, the level of viremia, the time to appearance of the standard serological markers, and the outcome of infection were indistinguishable from woodchucks transfected with wild-type WHV DNA. Furthermore, we could

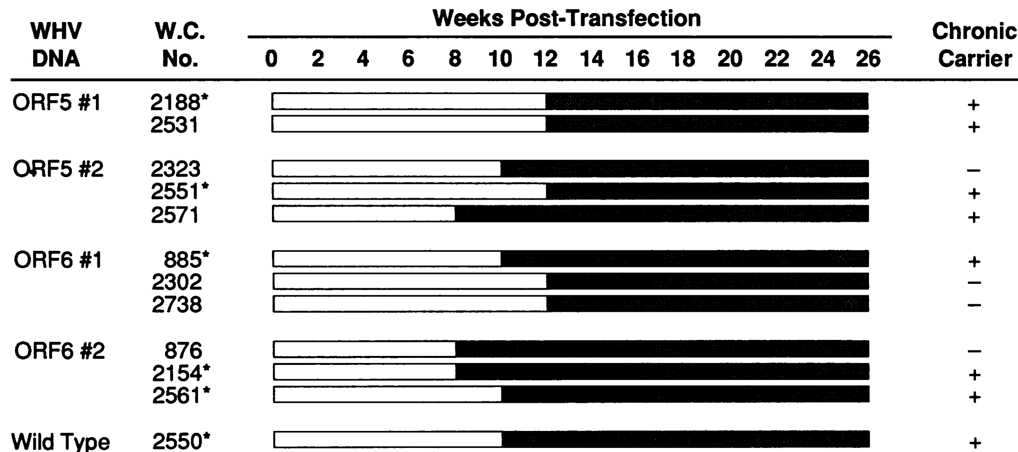

FIG. 2. Time of appearance of markers of WHV infection after transfection of woodchucks with ORF5 and ORF6 mutant DNA. One animal transfected with wild-type WHV DNA is also listed for comparison. Open boxes depict samples with no serological evidence of WHV infection, while shaded boxes depict samples with at least one marker of WHV infection (i.e., anti-WHc, WHsAg, or anti-WHs). Asterisks indicate the woodchucks (W.C.) that were WHsAg positive. Serum samples from these animals were used for PCR amplification and direct sequencing of WHV DNA. Only those woodchucks that were positive for WHsAg for >6 months were considered chronic carriers (+).

find no evidence for the presence of an antisense transcript (for expression of the ORF6 protein) in the livers of acutely or chronically infected woodchucks or for expression of a protein specific for ORF5 or ORF6 in in vitro translation experiments. Coupled with the fact that antibodies against the predicted proteins were absent in infected animals, we believe it is unlikely that ORF5 and ORF6 are authentic genes. The most important consequence of this finding is that now mutations can be made in the WHV genes that overlap these ORFs (i.e., X and polymerase) without concern for the introduction of extraneous lethal mutations.

One explanation for the conservation of ORF5 and ORF6 in the hepadnaviral genome is that these two ORFs may be vestigial gene sequences inherited from an ancestral virus. This hypothesis is supported by the finding of similar ORFs in the genomes of certain retroviruses that encode proteins involved in transactivation (*tat*/*tax*) of virus gene expression

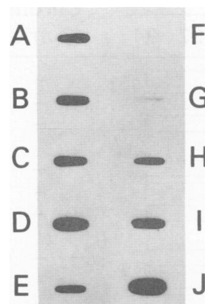

FIG. 3. Slot blot hybridization of WHV DNA. WHV DNA was extracted from 50-μl serum samples from chronic WHsAg carriers. Serum samples used in this study were collected at the peak of surface antigenemia. Extracted DNA was applied to a nitrocellulose membrane in alternative slots of a manifold. Slot A contains DNA from WC2188 (ORF5 mutant 1), slot B contains DNA from WC2551 (ORF5 mutant 2), slot C contains DNA from WC885 (ORF6 mutant 1), slot D contains DNA from WC2154 (ORF6 mutant 2, and slot E contains DNA from WC2550 (wild-type WHV8). Also shown is a titration of recombinant WHV8 DNA (9) in slots F to J with 5 (slot F), 10 (slot G), 50 (slot H), 100 (slot I), and 500 (slot J) pg of DNA.

(27). For example, the *tat*/*tax* gene region of human T-cell leukemia virus type I contains nine ORFs of ≥100 codons in length. Seven of the ORFs are located on the strand that contains the known virus genes. Two of the overlapping ORFs are known to be authentic genes. Most, if not all, retroviruses that encode a Tat/Tax protein possess this feature of multiple overlapping ORFs. Since hepadnaviruses and retroviruses appear to share a common evolutionary origin (15, 24, 27, 31, 33, 38, 46), it is likely that the feature of multiple, overlapping ORFs was inherited from an ancestral virus that gave rise to both hepadnaviruses and retroviruses. Although we cannot rule out the possibility that proteins are expressed at low levels from ORF5 and/or ORF6 in virus-infected cells, we believe that it is unlikely that such proteins play a major role in WHV replication.

The major goals of this study were to determine whether the X gene was necessary for the establishment of virus replication in the natural host and to define the important domains of the X protein using site-directed mutagenesis. A series of five X-gene mutants were constructed that changed the initiation codon to a codon specifying another amino acid (X mutant 1) or to truncate the carboxyl terminus of the X protein by 52, 31, 16, and 3 amino acids (X mutants 2 to 5, respectively). Only X mutant 5 was capable of independent replication in woodchucks. We found the following. (i) Mutation of the AUG initiation codon of the X gene to UUG

TABLE 2. Summary of X-gene mutants

X-gene mutant virus (map position of substitution[a])	Mutation	Changes in X gene or overlapping gene
1 (WHV 1503T)	ATG→TTG	X, initiation codon removed
2 (WHV 1771A)	TTG→TAG	X, truncated by 52 amino acids
3 (WHV 1833T)	CAA→TAA	X, truncated by 31 amino acids
4 (WHV 1879G)	TCA→TGA	X, truncated by 16 amino acids
5 (WHV 1917T)	CGA→TGA	X, truncated by 3 amino acids; precore, Ala→Val

[a] Map position of the single-nucleotide substitution with the unique *Eco*RI site as position 0 or 3323.

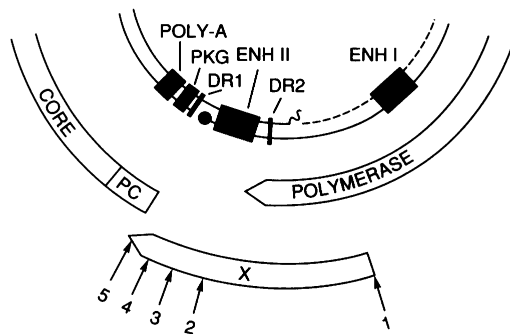

FIG. 4. WHV X-gene mutants. The positions of single-nucleotide substitutions in the WHV X gene are illustrated by arrows. X mutant 1 possesses an X gene that lacks an initiation codon, while X mutants 2 to 5 have premature termination codons introduced that result in truncation of the X protein by 52, 31, 16, and 3 amino acids, respectively. The abbreviations are defined in the legend to Fig. 1.

inhibited viral replication. (ii) The amino acid sequence between mutations 4 and 5 (amino acids 126 to 138; SIFV LGGCRHKCM) is crucial to the function of the X protein ($P = 0.0022$). (iii) The 3 amino acids at the extreme carboxyl terminus are not essential for the function of the X protein. These findings are in agreement with those of others who investigated the effect of mutation of the HBV X gene on its *trans*-activation potential. Removal of up to 12 amino acids from the carboxyl terminus of the HBV X protein (the HBV X protein extends 9 amino acids beyond that of WHV at the C terminus) did not change its *trans*-activation function in vitro (1, 19, 37, 49, 54), while the block of amino acids from positions 132 to 139, containing the highly conserved sequence FVLGGCRH, was shown to be essential for maintaining this function (1). Thus, it appears that this 8-amino-acid sequence is important for the function of the WHV and HBV X proteins.

However, our in vivo findings contrast with data that clearly show that a functional X gene is unnecessary for establishing a productive virus infection in hepatoma cell cultures transfected with HBV DNA (3, 16, 61). There are several possible explanations for these discordant findings. First, it could be argued that extraneous lethal mutations occurred by using our mutagenesis protocol and that these mutations, and not the X mutations, were responsible for the lack of replication in woodchucks. This explanation is unlikely, since such mutations are rare and we took the extra precaution of preparing pairs of identical mutants in parallel (see Materials and Methods). Animals were transfected with a mixture of equal amounts of the two identical mutants in quantities of DNA that should have resulted in a productive infection (e.g., 25 μg) if only one was infectious. In addition, we determined the complete nucleotide sequence of one of the mutant pairs for X mutants 1 and 3 and found that there were no changes in other regions of the two genomes. Second, one could postulate that the transfection procedure was ineffective in delivering DNA into the livers of the woodchucks. This is unlikely, since seven of seven animals transfected with WHV8 DNA in this and in previous experiments became productively infected, as did three of three animals possessing the most minor change in the WHV X gene in this experiment. Third, it is possible that the mutations made in the X gene had an adverse effect on an overlapping gene or embedded *cis*-acting element. In this regard, we made only single-nucleotide substitutions that avoided changing overlapping genes and were especially careful to avoid known regulatory sequences. In fact, we performed a multiple sequence alignment prior to this study to map conserved nucleotides in the mammalian hepadnavirus genome and avoided changing invariant nucleotides. Thus, the most likely explanation for the lack of replication of X mutants 1 to 4 is that the X gene is important for the establishment of a productive infection in woodchucks transfected with WHV DNA ($P < 0.01$ by Fisher exact test).

There are several questions that remain unanswered. The first is whether the WHV X-gene mutants that we have generated are capable of replication in cell culture. This question is not yet answerable, since we have been unsuccessful in propagating wild-type WHV8 DNA in HepG2 and Huh-7 cells, as well as primary woodchuck hepatocytes. In contrast, we readily detect evidence of viral replication, both

WHV DNA	W.C. No.	Weeks Post-Transfection: 0 2 4 6 8 10 12 14 16 18 20 22 24	Chronic Carrier
X # 1	892		–
	2406		–
	2745		–
X # 2	2718		–
	2734		–
	2832		–
X # 3	2435		–
	2706		–
	2918		–
X # 4	2556		–
	2712		–
	2742		–
X # 5	2831		–
	2865		–
	2873		+

FIG. 5. Time of appearance of markers of virus infection in woodchucks transfected with WHV X-gene mutant DNA. Open boxes depict samples with no serological evidence of WHV infection, while shaded boxes depict samples with at least one marker of WHV infection (i.e., anti-WHc, WHsAg, or anti-WHs). W.C., woodchuck; +, chronic carrier.

production of HBV surface antigen and particles possessing HBV DNA in the culture medium when we transfect these cells with HBV DNA. The most logical explanation for these results is that the level of WHV replication is significantly lower than that of HBV in these cells and is below our detection limits. In this regard, experiments are in progress to increase the sensitivity of detection of virus replication and to investigate whether other cell lines have the ability to replicate WHV at higher levels. A second important issue is to determine whether there is a correlation between the ability of a genome containing a mutant X gene to replicate and the ability of the mutant X protein to stimulate virus gene expression. To address this issue, we are in the process of generating recombinants that express wild-type or mutant X proteins and determining their ability to transactivate heterologous and homologous viral promoters in the chloramphenicol acetyltransferase assay. The results of these experiments may yield valuable insight into the function of the X protein in viral replication.

It is known that the HBV X protein does not bind directly to DNA but instead forms a protein-protein complex with cellular transcriptional factors (e.g., CREB, ATF-2, AP-2, etc.) and modifies their ability to bind to the enhancer of HBV (20, 43, 54). Seto et al. (42) showed that transactivation by the HBV X protein is cell specific but not species specific and suggested that the protein acts via cellular factors that are phylogenetically conserved and developmentally regulated. Koike et al. (16) demonstrated that both viral mRNA and production of core particles in HBV X mutants reached wild-type levels in Huh-7 cells but were markedly reduced in HepG2 cells. Taken together, it is reasonable to hypothesize that the X protein may not be required for virus replication in certain transformed cell lines but is important to initiate, or maintain, replication in the highly differentiated hepatocytes of the natural host.

ACKNOWLEDGMENTS

We thank L. Maloy for assistance in synthesis of oligopeptides and generation of rabbit antisera, T. Tsareva for assistance in oligonucleotide synthesis and purification, B. Baldwin for woodchuck studies, K. Cass, for serological testing, D. W. Alling for statistical assistance, and T. Heishman for editorial assistance. Computer analysis was supported by the GenBank Online Service.

This work was supported in part by contract numbers NO1-AI-72623 and NO1-AI-82698 from the National Institute of Allergy and Infectious Diseases to Georgetown and Cornell Universities, respectively.

REFERENCES

1. **Arii, M., S. Takada, and K. Koike.** 1992. Identification of three essential regions of hepatitis B virus X protein for transactivation function. Oncogene **7:**397–403.
2. **Aufiero, B., and R. J. Schneider.** 1990. The hepatitis B virus X-gene product trans-activates both RNA polymerase II and III promoters. EMBO J. **9:**497–504.
3. **Blum, H. E., Z.-S. Zhang, E. Galun, F. von Weizsacker, B. Garner, T. J. Liang, and J. R. Wands.** 1992. Hepatitis B virus X protein is not central to the viral life cycle in vitro. J. Virol. **66:**1223–1227.
4. **Blumberg, B. S., H. J. Alter, and S. Visnich.** 1965. A "new" antigen in leukemia sera. JAMA **191:**541–546.
5. **Colgrove, R., G. Simon, and D. Ganem.** 1989. Transcriptional activation of homologous and heterologous genes by the hepatitis B virus X gene product in cells permissive for viral replication. J. Virol. **63:**4019–4026.
6. **Cote, P. J., R. E. Engle, C. A. Langer, A. Ponzetto, and J. L. Gerin.** 1984. Antigenic analysis of woodchuck hepatitis virus surface antigen with site-specific radioimmunoassays. J. Virol. **49:**701–708.
7. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
8. **Feitelson, M. A., I. Millman, T. Halbherr, H. Simmons, and B. S. Blumberg.** 1986. A newly identified hepatitis B type virus in tree squirrels. Proc. Natl. Acad. Sci. USA **83:**2233–2237.
9. **Ganem, D., and H. E. Varmus.** 1987. The molecular biology of the hepatitis B viruses. Annu. Rev. Biochem. **56:**651–693.
10. **Gerin, J. L., P. J. Cote, B. E. Korba, and B. C. Tennant.** 1989. Hepadnavirus-induced liver cancer in woodchucks. Cancer Detect. Prev. **14:**227–229.
11. **Girones, R., P. J. Cote, W. E. Hornbuckle, B. C. Tennant, J. L. Gerin, R. H. Purcell, and R. H. Miller.** 1989. Complete nucleotide sequence of a molecular clone of woodchuck hepatitis virus that is infectious in the natural host. Proc. Natl. Acad. Sci. USA **86:**1846–1849.
12. **Gust, I. D., C. J. Burrell, A. G. Coulepis, W. S. Robinson, and A. J. Zuckerman.** 1986. Taxonomic classification of human hepatitis B virus. Intervirology **25:**14–29.
13. **Kaneko, S., S. M. Feinstone, and R. H. Miller.** 1989. Rapid and sensitive method for the detection of serum hepatitis B virus DNA using the polymerase chain reaction technique. J. Clin. Microbiol. **27:**1930–1933.
14. **Kaneko, S., and R. H. Miller.** 1988. X-region-specific transcript in mammalian hepatitis B virus-infected liver. J. Virol. **62:**3979–3984.
15. **Khudyakov, Y. E., and A. M. Makhov.** 1989. Prediction of terminal protein and ribonuclease H domains in the gene P product of hepadnaviruses. FEBS Lett. **243:**115–118.
16. **Koike, K., Y. Shirakata, K. Yaginuma, M. Arii, S. Takada, I. Nakamura, Y. Hayashi, M. Kawada, and M. Kobayashi.** 1989. Oncogenic potential of hepatitis B virus. Mol. Biol. Med. **6:**151–160.
17. **Korba, B. E., P. J. Cote, F. V. Wells, B. Baldwin, H. Popper, R. H. Purcell, B. C. Tennant, and J. L. Gerin.** 1989. Natural history of woodchuck hepatitis virus infections during the course of experimental viral infection: molecular virologic features of the liver and lymphoid tissues. J. Virol. **63:**1360–1370.
18. **Kwee, L., R. Lucito, B. Aufiero, and R. J. Schneider.** 1992. Alternate translation initiation on hepatitis B virus X mRNA produces multiple polypeptides that differentially transactivate class II and III promoters. J. Virol. **66:**4382–4389.
19. **Levrero, M., C. Balsano, G. Natoli, M. L. Avantaggiati, and E. Elfassi.** 1990. Hepatitis B virus X protein transactivates the long terminal repeats of human immunodeficiency virus type 1 and 2. J. Virol. **64:**3082–3086.
20. **Maguire, H. F., J. P. Hoeffler, and A. Siddiqui.** 1991. HBV X protein alters the DNA binding specificity of CREB and ATF-2 by protein-protein interactions. Science **252:**842–844.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **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 humans. Proc. Natl. Acad. Sci. USA **77:**2941–2945.
23. **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.
24. **Miller, R. H.** 1988. Close evolutionary relatedness of the hepatitis B virus and murine leukemia virus polymerase gene sequences. Virology **164:**147–155.
25. **Miller, R. H.** 1988. Human immunodeficiency virus may encode a novel protein on the genomic DNA plus strand. Science **239:**1420–1422.
26. **Miller, R. H.** 1990. Organization of the X gene region of the hepatitis B virus genome. Gastroenterol. Jpn. **25**(Suppl. 2)**:**1–5.
27. **Miller, R. H.** 1991. Evolutionary relationship between hepadnaviruses and retroviruses, p. 227–244. *In* A. McLachlan (ed.), Molecular biology of the hepatitis B virus. CRC Press, Boca Raton, Fla.

28. **Miller, R. H., R. Girones, P. J. Cote, W. E. Hornbuckle, T. Chestnut, B. H. Baldwin, B. E. Korba, B. C. Tennant, J. L. Gerin, and R. H. Purcell.** 1990. Evidence against a requisite role for defective virus in the establishment of persistent hepadnavirus infection. Proc. Natl. Acad. Sci. USA **87**:9329–9332.
29. **Miller, R. H., S. Kaneko, C. T. Chung, R. Girones, and R. H. Purcell.** 1989. Compact organization of the hepatitis B virus genome. Hepatology **9**:322–327.
30. **Miller, R. H., S.-C. Lee, Y.-F. Liaw, and W. S. Robinson.** 1985. Hepatitis B viral DNA in infected human liver and in hepatocellular carcinoma. J. Infect. Dis. **151**:1081–1092.
31. **Miller, R. H., P. L. Marion, and W. S. Robinson.** 1984. Hepatitis B viral DNA-RNA hybrid molecules in particles from infected liver are converted to viral DNA molecules during an endogenous DNA polymerase reaction. Virology **139**:64–72.
32. **Miller, R. H., and W. S. Robinson.** 1984. Hepatitis B virus DNA forms in nuclear and cytoplasmic fractions of infected human liver. Virology **137**:390–399.
33. **Miller, R. H., and W. S. Robinson.** 1986. Common evolutionary origin of hepatitis B virus and retroviruses. Proc. Natl. Acad. Sci. USA **83**:2531–2535.
34. **Moroy, T. J., C. Etiemble, C. Trepo, P. Tiollais, and M.-A. Buendia.** 1985. Transcription of woodchuck hepatitis virus in the chronically infected liver. EMBO J. **4**:1507–1514.
35. **Nakamaye, K. L., and F. Eckstein.** 1986. Inhibition of restriction endonuclease NciI cleavage by phosphorothioate groups and its application to oligonucleotide-directed mutagenesis. Nucleic Acids Res. **14**:9679–9698.
36. **Ponzetto, A., P. J. Cote, E. C. Ford, R. Engle, J. Cicmanec, M. Shapiro, R. H. Purcell, and J. L. Gerin.** 1985. Radioimmunoassay and characterization of woodchuck hepatitis virus core antigen and antibody. Virus Res. **2**:301–315.
37. **Ritter, S. E., T. M. Whitten, A. T. Quets, and R. H. Schloemer.** 1991. An internal domain of the hepatitis B virus X antigen is necessary for transactivating activity. Virology **182**:841–845.
38. **Robinson, W. S., R. H. Miller, and P. L. Marion.** 1987. Hepadnaviruses and retroviruses share genome homology and features of replication. Hepatology **7**:64S–73S.
39. **Rossner, M. T.** 1992. Review: hepatitis B virus X-gene product: a promiscuous transcriptional activator. J. Med. Virol. **36**:101–117.
40. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
41. **Seto, E., P. J. Mitchell, and T. S. B. Yen.** 1990. Transactivation by the hepatitis B virus X protein depends on AP-2 and other transcription factors. Nature (London) **344**:72–74.
42. **Seto, E., D.-X. Zhou, B. M. Peterlin, and T. S. B. Yen.** 1989. Trans-activation by the hepatitis B virus X protein shows cell-type specificity. Virology **173**:764–766.
43. **Siddiqui, A., R. Gaynor, A. Srinivasan, J. Mapoles, and R. W. Farr.** 1989. Trans-activation of viral enhancers including long terminal repeat of the human immunodeficiency virus by the hepatitis B virus X protein. Virology **169**:479–484.
44. **Spandau, D. F., and C.-H. Lee.** 1988. *trans*-Activation of viral enhancers by the hepatitis B virus X protein. J. Virol. **62**:427–434.
45. **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.
46. **Summers, J., and W. S. Mason.** 1982. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell **29**:403–415.
47. **Summers, J., J. M. Smolec, and R. Snyder.** 1978. A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. Proc. Natl. Acad. Sci. USA **75**:4533–4537.
48. **Tagawa, M., M. Omata, and P. L. Marion.** 1990. Open reading frames on plus strand genome of duck hepatitis B virus. Gastroenterol. Jpn. **25**(Suppl. 2):20–22.
49. **Takada, S., and K. Koike.** 1990. Trans-activation function of a 3′ truncated X gene-cell fusion product from integrated hepatitis B virus DNA in chronic hepatitis tissues. Proc. Natl. Acad. Sci. USA **87**:5628–5632.
50. **Takada, S., and K. Koike.** 1990. X protein of hepatitis B virus resembles a serine protease inhibitor. Jpn. J. Cancer Res. **81**:1191–1194.
51. **Twu, J.-S., and W. S. Robinson.** 1989. Hepatitis B virus X gene can transactivate heterologous viral sequences. Proc. Natl. Acad. Sci. USA **86**:2046–2050.
52. **Twu, J.-S., and R. H. Schloemer.** 1987. Transcriptional *trans*-activating function of hepatitis B virus. J. Virol. **61**:3448–3453.
53. **Twu, J.-S., J. Y. Wu, and W. S. Robinson.** 1990. Transcriptional activation of the human immunodeficiency virus type 1 long terminal repeat by hepatitis B virus X-protein requires de novo protein synthesis. Virology **177**:406–410.
54. **Unger, T., and Y. Shaul.** 1990. The X protein of the hepatitis B virus acts as a transcription factor when targeted to its responsive element. EMBO J. **9**:1889–1895.
55. **Wang, W., W. T. London, and M. A. Feitelson.** 1991. Hepatitis B x antigen in hepatitis B virus carrier patients with liver cancer. Cancer Res. **51**:4971–4977.
56. **Wang, W., W. T. London, L. Lega, and M. A. Feitelson.** 1991. HBxAg in the liver from carrier patients with chronic hepatitis and cirrhosis. Hepatology **14**:29–37.
57. **Winship, P. R.** 1989. An improved method for directly sequencing PCR amplified material using dimethyl sulfoxide. Nucleic Acids Res. **17**:1266.
58. **Wollersheim, M., U. Debelka, and P. H. Hofschneider.** 1988. A transactivating function encoded in the hepatitis B virus X gene is conserved in the integrated state. Oncogene **3**:545–552.
59. **Wong, D. C., J. W.-K. Shih, R. H. Purcell, J. L. Gerin, and W. T. London.** 1982. Natural and experimental infection of woodchucks with woodchuck hepatitis virus, as measured by new, specific assays for woodchuck surface antigen and antibody. J. Clin. Microbiol. **15**:484–490.
60. **Wu, J. Y., Z.-Y. Zhou, A. Judd, C. A. Cartwright, and W. S. Robinson.** 1990. The hepatitis B virus-encoded transcriptional trans-activator hbx appears to be a novel protein serine/threonine kinase. Cell **63**:687–695.
61. **Yaginuma, K., Y. Shirakata, M. Kobayashi, and K. Koike.** 1987. Hepatitis B virus (HBV) particles are produced in a cell culture system by transient expression of transfected HBV DNA. Proc. Natl. Acad. Sci. USA **84**:2678–2682.