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The Precore Gene of the Woodchuck Hepatitis Virus Genome Is Not Essential for Viral Replication in the Natural Host

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A number of naturally occurring hepatitis B virus mutants that cannot synthesize the virus precore protein have been identified. Such mutants have been associated with more severe forms of hepatitis, including fulminant hepatitis. The most common mutation observed is a substitution of G to A in the distal precore gene that converts a codon specifying Trp (TGG) to a termination codon (TAG). Using oligonucleotide-directed mutagenesis, we have produced the same point mutation in the precore gene of an infectious clone of woodchuck hepatitis virus (WHV). Transfection of mutant WHV DNA into the livers of adult woodchucks resulted in replication of the mutant in three of three susceptible animals. Levels of virus replication and transient elevations in liver enzymes in serum were similar to those of adult animals infected with wild-type WHV. Virions, found to possess mutant precore genes by polymerase chain reaction amplification and DNA sequencing, were recovered from the serum of one of the animals and inoculated subcutaneously into neonatal woodchucks. They produced infection in all five animals studied. The level of virus replication in neonatal animals infected with this mutant virus was comparable to that found in neonatal woodchucks infected with wild-type WHV, but none of five woodchucks infected with the precore mutant virus as neonates became chronic virus carriers. It was concluded that the precore gene of the WHV genome is not essential for virus replication in the natural host but may be important for chronic infection.

Woodchucks (*Marmota monax*) infected with woodchuck hepatitis virus (WHV) provide a valuable animal model system for the study of hepatitis B virus (HBV) infection of humans (3, 4, 7, 8, 14). The WHV and HBV genomes are organized in a similar fashion and have approximately 65% nucleotide sequence homology. WHV produces acute and/or chronic hepatitis in woodchucks, similar to HBV infections of humans, and virtually all chronically infected woodchucks develop hepatocellular carcinoma in 2 to 3 years (3, 14). HBV infection in humans often progresses to hepatocellular carcinoma in 20 to 30 or more years. Thus, WHV infection of woodchucks represents a useful model system for studying the pathogenesis of HBV disease.

The core gene of hepadnaviruses is usually preceded by an in-phase precore gene with a separate AUG initiation codon. The precore gene encodes a signal sequence that directs the precore protein to a membranous compartment for proteolytic processing, glycosylation, and extracellular secretion as e antigen (13, 16). Clinically, the presence of hepatitis B e antigen (HBeAg) in the serum of infected individuals is correlated with the presence of active viral replication. Normally, seroconversion from HBeAg to anti-HBe is associated with remission of liver injury and clearance of serum HBV DNA (11). In Japan and several Mediterranean countries, it has been found that a spontaneously occurring point mutation of G to A in the distal precore gene of HBV leads to the premature termination of the precore protein, the precursor of HBeAg, and that this change is associated with acute fulminant or severe chronic hepatitis in some patients (12). In some areas, 10% of anti-HBe-positive hepatitis patients have persistent viral replication and chronic hepatitis (1, 11, 18), and it has been proposed that the inability of the mutant virus to produce the precore protein is related to more severe hepatic disease in infected individuals.

Although there is no documented proof for the existence of an e antigen in the sera of WHV-infected woodchucks, it is likely to exist, because this protein is encoded by the two most divergent hepadnaviruses, HBV and duck HBV. Previous studies have shown that the precore gene in the duck HBV genome is required for synthesis and secretion of e antigen but not for replication in vivo (2, 16). Also, a spontaneously occurring HBV mutant containing a nonsense mutation in the distal precore gene was found to be replication competent in tissue culture cells (i.e., HepG2 cells) without expressing the 17-kDa HBeAg (17). However, there are limited data on the infectivity of the recombinant HBV DNA containing the precore mutation in chimpanzees (18). Thus, it is not known whether mammalian hepadnavirus DNA with a defective precore gene is universally infectious when transfected into the liver of the natural host.

Since the amino acids of the precore protein of HBV and WHV are well conserved, we were able to reproduce in WHV the exact mutation identified in HBV precore mutants (Fig. 1A and B). The infectious WHV8 genome (4) was subcloned into the phagemid pBluescript II KS(+) (Stratagene, La Jolla, Calif.), and single-stranded DNA templates were generated for mutagenesis with helper phage VCS M13 (Stratagene). The mutation was made by the method of Nakamaye and Eckstein (10) using oligonucleotide-directed in vitro mutagenesis (Amersham, Arlington Heights, Ill.). Briefly, a mutagenic oligonucleotide containing a substitu-

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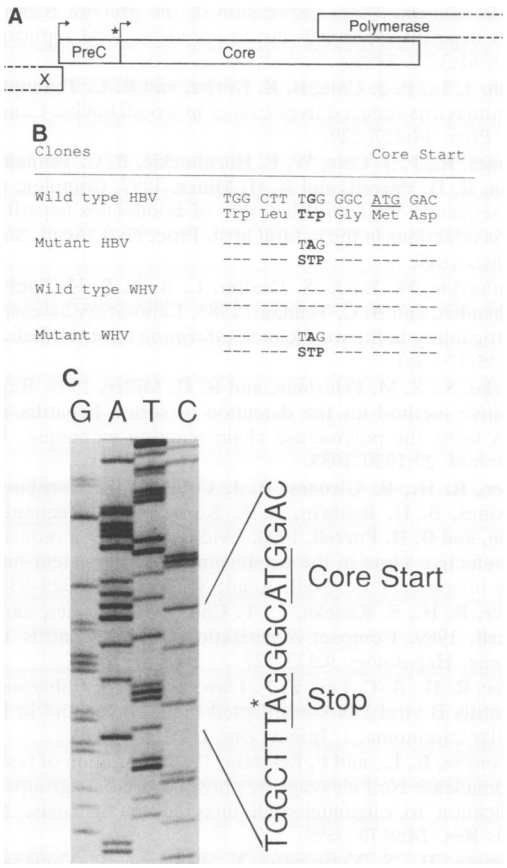

FIG. 1. Construction of the WHV precore mutant. (A) Organization of the precore and core gene regions of the WHV genome. →, ATG codons in the precore (PreC) and core genes; *, site of precore mutation. (B) Comparison of the nucleotide and deduced amino acid sequences of the distal precore region in wild-type and precore mutant HBV and WHV. STP, stop codon; dashes represent identical nucleotide and amino acid sequences. (C) Nucleotide sequence of the distal precore gene from the WHV precore mutant genome.

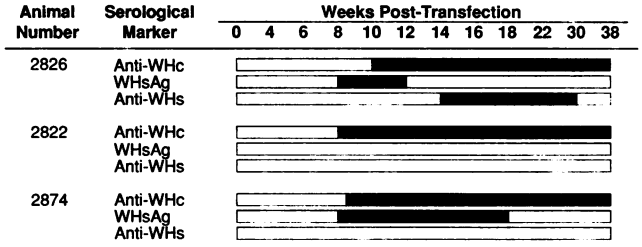

FIG. 2. Serological profile of three woodchucks transfected with WHV precore mutant DNA. Serial semimonthly serum samples were assayed for serological evidence of infection by enzyme-linked immunosorbent assay and solid phase radioimmunoassay. Negative results are shown as open boxes, while positive results are shown as shaded boxes.

tion of G to A was annealed to the 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 the 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 polymerase I and T4 DNA ligase reactions and were used to transform competent *Escherichia coli*. Mutated WHV clones were identified by DNA sequencing (15). One clone was selected for further investigation, and the complete nucleotide sequence of the 3.3-kb genome was determined to verify that only one nucleotide in the genome was altered (Fig. 1C).

Mutant WHV DNA was prepared for transfection into the livers of adult woodchucks by release of WHV DNA from the vector genome by cleavage with restriction endonuclease *Eco*RI, and linear WHV DNA was purified by agarose gel electrophoresis and electroelution. Linear WHV genomes were ligated at concentrations (5 to 10 μg/ml) optimal for production of monomeric circular genomes (7). WHV precore mutant DNA was resuspended in nonpyrogenic phosphate-buffered saline and injected directly into the surgically exposed livers of adult woodchucks (7). Three adult woodchucks were used in this transfection study, and each animal received 50 μg of recombinant WHV DNA in a volume of 0.5 ml injected at multiple sites. Serological assays of the semimonthly serum samples revealed that all three woodchucks became positive for one or more markers of WHV infection approximately 8 weeks after transfection (Fig. 2). To verify that serum-derived virions contained the mutant WHV genome, WHV DNA from the serum of animals positive for WHV surface antigen was amplified in a nested polymerase chain reaction assay (6) and the nucleotide sequence of the precore gene was determined by the dideoxy method in the presence of dimethyl sulfoxide (19). Analysis indicated that the nucleotide sequence of the entire precore gene of the genomes from the transfected animals was identical to the input mutant DNA used for in situ hepatic transfection (data not shown). We conclude that the intact precore gene is not essential for WHV replication in its natural host.

Next, we analyzed the levels of virus DNA and liver enzymes in the sera of the transfected woodchucks. First, the levels of WHV DNA in the sera of woodchuck 2826 (WC2826) and WC2874 were found to be similar by a semiquantitative polymerase chain reaction assay. Next, WHV DNA was extracted from the serum of WC2874 during the peak of surface antigenemia and quantified by slot blot hybridization analysis with a ^{32}P-labeled WHV DNA probe (9). The level of virus DNA, determined by autoradiography, was approximately 10 ng/ml, which was within the range normally seen in animals infected with wild-type WHV (0.5 to 20 ng/ml).

The serum sorbitol dehydrogenase (SDH) activity has been shown to be a useful marker of hepatocellular injury in woodchucks (5). Prior to transfection, the mean serum SDH activity of the three woodchucks was 15 ± 2 IU/liter (mean ± standard deviation). Ten weeks following transfection, significant elevations of SDH activity were observed in two of three animals, WC2874 and WC2826. The level of SDH remained elevated for 3 and 5 weeks, with maximum values of 648 and 192 IU/liter, respectively. These results were similar to those observed for eight adult woodchucks that were experimentally infected with wild-type WHV by intravenous inoculation of $\approx 1 \times 10^7$ infectious doses of virus (1 infectious dose $\approx$1 genome). The mean value of SDH in serum before inoculation of this group was 14 ± 3 IU/liter. Significant elevations of SDH activity were observed in five of the eight woodchucks beginning 7 to 10 weeks following

inoculation, and SDH activity in serum remained elevated for 2 to 6 weeks. The mean maximum SDH activity in serum in woodchucks infected with wild-type WHV was 301 ± 110 IU/liter, with a range of 136 to 730 IU/liter. Hepatic tissue was not histologically examined. Taken together, these data suggest that the level of virus replication and of liver injury caused by infection with the WHV precore mutant are similar to those of woodchucks infected with wild-type virus.

Finally, we demonstrated the infectivity of the mutant WHV virions derived from serum. WC2874 became positive for WHV surface antigen by week 8 (Fig. 2), and additional serum was collected 2 days later. Five 3-day-old woodchuck pups from three different litters were inoculated subcutaneously with 100 μl (3×10^8 to 4×10^8 genomes) of the WHV-positive serum from WC2874 and subsequently were monitored for serological evidence of infection. All five animals became positive for one or more markers of WHV infection 2 to 4 months after inoculation (data not shown). Slot blot hybridization analysis of serum WHV DNA, extracted from the WHV surface antigen-positive serum samples, revealed a level of WHV of approximately 0.5 to 4 ng/ml, which is within the range seen in wild-type WHV-infected neonates (i.e., 0.5 to 20 ng/ml). Within the parameters examined, infection of susceptible woodchucks by WHV with a defective precore gene was indistinguishable from a wild-type WHV infection.

It is noteworthy that none of the five infected neonatal woodchucks became chronic carriers of the virus. This result is significantly different from that from a recent study of 20 woodchucks infected at 3 days of age with virus generated from the parental cDNA clone (i.e., WHV8). In the latter study, 14 (70%) of the woodchucks became chronic carriers (7). Before it is concluded that the reduction in chronic carrier rate is due to biological changes caused by the defective precore gene, additional experiments must be performed.

Although our data on the chronic carrier rate of neonatal woodchucks infected with precore mutant WHV are preliminary, this finding supports the hypothesis by Brunetto and coworkers (1) that the HBV precore mutants may be unable to induce chronic infection without the helper function of wild-type HBV. This hypothesis can now be tested in the woodchuck model system. Future experiments will address the importance of the WHV precore gene in the biology of virus infection. It is clear, however, from the present study that the WHV precore gene is not essential for virus replication in the natural host.

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