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Evidence against a requisite role for defective virus in the establishment of persistent hepadnavirus infections

(hepatitis B virus/woodchuck hepatitis virus/virus mutants/chronic virus infection/animal model)

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ABSTRACT **The factors involved in the establishment of persistent hepadnavirus infection are poorly understood. Recent findings demonstrate that the sequence of the genome of hepatitis B virus (HBV) is variable in infected individuals and that, in some cases, virus mutants predominate. Our objectives in the present study were to analyze the variability of woodchuck hepatitis virus (WHV) genomes in an infected animal and to determine whether sequence heterogeneity played a critical role in the ability of WHV to induce chronic infection. We cloned and determined the complete nucleotide sequence of three supercoiled genomes from an animal that became infected after inoculation with a standardized WHV serum pool (i.e., the WHV7 virus pool). We found that there were four nucleotide substitutions among the three genome sequences as well as a 73-nucleotide deletion in one of the recombinants. DNA transfection experiments revealed that only one of the three recombinants was capable of independent replication. These data suggest that a significant proportion of replicative templates in woodchucks that are infected with WHV are defective virus genomes. Next, we compared the outcome of acute infection after inoculation with a serum pool containing a uniform population of replication competent virus (i.e., the WHV7R pool) with a serum pool composed of WHV genomes of variable sequence. The WHV7R serum pool originated from a woodchuck that became a chronic carrier after *in vivo* transfection of the liver with the infectious WHV7 recombinant. Neonatal woodchucks were inoculated with 5×10^6 WHV genome equivalents of either the WHV7 pool or the WHV7R pool. All animals in the study became acutely infected with WHV. Of the animals infected with the WHV7 serum pool, 65% became chronic carriers, while 80% of the animals infected with the WHV7R serum pool developed chronic infection. Thus, infection of woodchucks with a serum pool containing defective virus resulted in a rate of chronic WHV infection that was similar to, or even lower than, a rate from a pool containing only wild-type virus. This suggests that the presence of defective virus in the inoculum is not a prerequisite for the establishment of persistent hepadnavirus infections.**

Hepatitis B virus (HBV), a member of the hepadnavirus family, is an important human pathogen that persistently infects ≈300 million people worldwide. Our previous investigations (1), and those of others (2, 3), have demonstrated that there is sequence variation among virus genomes isolated from individual patients infected with HBV. Recent results indicate that mutant virus genomes, with lesions in the precore or core gene regions, are found in some patients persistently infected with HBV (4–10). Therefore, it is possible that defective virus plays a role in the establishment of chronic hepadnavirus infections.

Woodchuck hepatitis virus (WHV) infection of woodchucks has proven to be an excellent animal model system for the study of HBV infection of humans. The complete genome sequence of five WHV isolates has been published (11–14) and the recombinant DNA genome of at least one of the isolates is known to be infectious in woodchuck liver transfection experiments (14). A number of studies have been performed to characterize the biology of WHV infection, and it is clear that woodchucks infected experimentally with WHV develop acute virus infections, which can become chronic infections leading to hepatocellular carcinoma (15–21). We have found that inoculation of neonatal woodchucks with 5×10^6 WHV genome equivalents of the WHV7 serum pool produces acute infection in all animals tested. A total of 35% of these animals developed antibody against WHV surface antigen (anti-WHsAg) by the 5th month postinoculation, while 65% became chronic WHV carriers for life (22). Our recent findings, presented here, indicate that WHV supercoiled genomes cloned from an infected woodchuck are variable in sequence and that the majority of the recombinants are defective mutants. In addition, we found that the development of chronic infection did not require sequence variation within the WHV inoculum.

MATERIALS AND METHODS

Cloning and Sequence Analysis of Supercoiled WHV7 DNA from an Infected Woodchuck. Woodchuck 1481 (WC 1481) was inoculated with the WHV7 serum pool at 3 days of age. The animal became infected with WHV and was sacrificed 3 months postinoculation. WHV7 supercoiled DNA was isolated from hepatocyte nuclei and was cloned into the *Eco*RI restriction endonuclease site of vector pUC13 (Pharmacia) as described (13, 23). Three independent recombinants were selected for analysis and were designated pWHV7-3, -9, and -11. The complete nucleotide sequence of pWHV7-9 DNA has been published under the designation WHV7 (13). In this study, the complete nucleotide sequence of pWHV7-3 and -11 DNA was determined by the dideoxynucleotide sequencing method and was compared to the sequence of pWHV7-9 (13).

Fidelity of the Replication of the WHV Genome in *Escherichia coli* During Molecular Cloning Experiments. Although it is unlikely that base substitutions occur during propagation of recombinant genomes in *E. coli*, because of the high fidelity

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Abbreviations: HBV, hepatitis B virus; WHV, woodchuck hepatitis virus; WHsAg, woodchuck hepatitis surface antigen; WHcAg, woodchuck hepatitis core antigen; HBeAg, HBV e antigen.

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of the replication of plasmid genomes by a polymerase that possesses proofreading functions, we were concerned about the possible existence of hot spots of mutation within the WHV genome. Therefore, we examined the fidelity of the replication of the WHV genome in *E. coli*. WHV plasmid pWHV8 (14) was propagated in *E. coli* strain LE392 in the following manner: (*i*) Cells were taken from the original frozen stock stored at −70°C and placed in 10 ml of fresh Luria broth (LB) with the appropriate antibiotic and grown overnight at 37°C with constant shaking. (*ii*) A 1-ml sample of the fresh overnight stock was used to inoculate 1 liter of LB plus antibiotic, and the bacteria were grown overnight at 37°C with constant shaking. This overnight culture was designated as the P1 culture. (*iii*) A 1-ml sample was taken from the P1 culture and was used to inoculate a second flask containing 1 liter of LB plus antibiotic. (*iv*) This process was repeated 25 times (i.e., to P25) representing ≈250 doublings of the bacteria. (*v*) Bacteria from the P25 culture were plated and two independent colonies, designated P25-A and -B, were selected for further analysis. Plasmid DNA was isolated from P25-A and -B and from the bacteria from the P1 culture, and the nucleotide sequence of the WHV DNA was determined as described above.

Transfection of Woodchucks with Recombinant WHV DNA. Recombinant WHV7 DNA was prepared for transfection by digestion of pWHV7 DNA with the restriction endonuclease *Eco*RI, and separation of WHV7 DNA from pUC13 DNA sequences by agarose gel electrophoresis and electroelution (14). The recombinant WHV7 genomes were ligated at a concentration of 5–10 μg/ml to form monomeric, circular molecules. The ligated DNA was alcohol precipitated and the pellet was resuspended in phosphate-buffered saline to a final concentration of 10 μg of DNA per 50 μl. A 50-μl aliquot of DNA was transfected into the liver of individual neonatal woodchucks (3–7 days old) as described (14). WHsAg and antibody against WHV core antigen (anti-WHcAg) were assayed by RIA (24, 25). Anti-WHsAg was detected by a solid-phase RIA (26).

Infection of Neonatal Woodchucks with WHV7 and WHV7R Serum Pools. The WHV7 serum pool was derived from WC 7, a wild-caught woodchuck that was a chronic WHV carrier that ultimately developed hepatocellular carcinoma (27). The WHV7R serum pool was derived from WC 2854, a woodchuck that had been transfected with recombinant pWHV7-11 DNA 10 months previously. The number of virus genomes per ml of serum was determined by slot blot hybridization. The pools were diluted to contain 5×10^6 WHV genomes per 0.1 ml of serum. Neonatal woodchucks 1–7 days old were inoculated subcutaneously with 0.1 ml of either the WHV7 or WHV7R serum pool. Animals were bled and tested for serological markers of WHV infection monthly for 9 months and quarterly thereafter.

RESULTS

To determine the degree of genome variation among WHV replicative intermediates, we isolated supercoiled molecules from the nuclei of liver cells of a woodchuck infected with WHV7. The supercoiled DNA was cloned and the complete nucleotide sequence of three individual recombinants, designated pWHV7-3, -9, and -11, was determined. We noted several inconsistencies in the nucleotide sequence of WHV7-9 DNA compared to our previously published WHV7 sequence. Specifically, we found that nucleotide 1485 is an A and not a G, and nucleotide 2331 is a C and not an A as reported (13). Comparison of the consensus genome sequence of the three WHV7 recombinants (Table 1) revealed that there were a total of five differences among the clones. There were 4 nucleotide substitutions among the recombinants as well as a 73-nucleotide deletion in one clone (i.e., pWHV7-3). Therefore, the supercoiled replicative intermediates of a woodchuck infected with WHV are heterogeneous in sequence.

It is unlikely that the changes among WHV7 recombinants arose during propagation of the recombinant plasmid because the bacterial polymerase possesses a proofreading function and replicates DNA with high fidelity. However, since it was possible that the virus genome contained sequences representing hot spots for mutation, we determined the fidelity of the replication of the recombinant WHV genome during passage in *E. coli*. We found that two progeny WHV plasmids that were passaged in bacteria for >250 bacterial cell divisions did not contain any nucleotide changes when compared to the parental plasmid DNA. Thus, we conclude that it is unlikely that the sequence of the WHV7 recombinants changed during propagation in *E. coli*.

Next, we determined the infectivity of the recombinant WHV7 DNAs. Transfection of monomeric, circular DNA into the liver of neonatal woodchucks demonstrated that only one of the three recombinants (i.e., pWHV7-11) was infectious (Table 2). The serological profile of two representative woodchucks transfected with pWHV7-11 is shown in Table 3. Both WC2850 and WC2854 became positive for WHsAg and anti-WHcAg 3 months posttransfection, a finding that is similar to the response of the other animals that became infected. WC2850 seroconverted to anti-WHsAg while WC2854 developed a persistent WHV infection. Overall, these data suggest that both wild-type (i.e., replication competent) and mutant (i.e., replication negative) WHV replicative intermediates are present in chronically infected woodchucks.

Next, we determined the rate of the development of chronic virus infection in animals inoculated with two different WHV7 serum pools. Several of the woodchucks transfected with pWHV7-11 DNA became persistently infected with WHV. Serum was collected from one such animal [i.e., WC 2854 (Table 3)] and was used to create the WHV7 recombinant (WHV7R) virus inoculum. Neonatal woodchucks were infected with 5×10^6 WHV genome equivalents of either the original WHV7 serum pool consisting of virions with variable genome sequences or the WHV7R serum pool derived from a single infectious genome. All animals from

Table 1. Nucleotide sequence differences among WHV7 supercoiled genomes isolated from a chronically infected woodchuck

Recombinant	Map unit	Gene	Change from consensus sequence	
			Nucleotide	Amino acid (codon)
pWHV7-11	184	Presurface	C → A	Arg (CGC) → Arg (CGA)
		Polymerase		Pro (CCA) → Thr (ACA)
pWHV7-9	1485	Polymerase	G → A	Gly (GGG) → Gly (GGA)
pWHV7-3	1904–	X	73-bp	8-residue deletion
	1976	Precore	deletion	16-residue deletion
pWHV7-11	2158	Core	A → G	Glu (GAA) → Glu (GAG)
pWHV7-9	2331	Core	A → C	His (CAT) → Pro (CCT)

bp, Base pair.

Table 2. Infectivity of recombinant WHV7 DNA

Recombinant	Neonatal woodchucks transfected	Woodchucks with serological evidence of WHV infection
pWHV7-3	12	0 (0%)
pWHV7-9	7	0 (0%)
pWHV7-11	11	10 (91%)

both study groups became infected with WHV. This indicates that the virions produced from the transfection of woodchucks with replication competent recombinant WHV DNA represent infectious virus. Interestingly, 65% of the animals infected with the WHV7 serum pool (22) and 80% (i.e., 16 of 20) of the animals infected with the WHV7R serum pool became persistently infected with WHV. The kinetics of virus infection of neonatal woodchucks using the WHV7R serum pool (Table 4) were similar to those found previously for the WHV7 serum pool (data not shown) and for the WHV7 transfection experiments (Table 3). These data indicate that infection of neonatal woodchucks with a serum pool consisting of wild-type virus, with a uniform genome sequence, results in a chronic carrier rate equal to, or possibly greater than, the rate observed after infection with a serum pool containing defective virus genomes.

DISCUSSION

Studies on the heterogeneity of the genome sequence of HBV have been performed in our laboratory (1) and in the laboratories of others (2, 3). The consensus finding is that there is variation among HBV genome sequences in infected patients. Evidence is accumulating that some chronic carriers of HBV possess mutant virus with defects in the precore or core genes (4–10). In some cases, mutant virus genomes outnumber wild-type virus genomes. For example, Carman *et al.* (7) found that 7 of 18 patients, who were positive for circulating HBV particles but negative for HBV e antigen (HBeAg), possessed HBV genomes with specific lesions in the precore gene region. In addition, Okamoto and coworkers (10) found that HBV mutants with defects in precore predominated in the serum of three patients as they seroconverted from HBeAg to anti-HBeAg. The most common mutation in the precore region was a point mutation that converted a codon that normally encodes tryptophan (i.e., TGG) to a termination codon (i.e., TAG). Thus, it is possible that mutant virus genomes play a role in anti-HBeAg-positive HBV chronic infections.

Table 3. Serological profile of woodchucks transfected with WHV7-11

Woodchuck	Months postinfection	Anti-WHcAg*	WHsAg†	Anti-WHsAg‡
2850	2	0.1	0.5	0.0
	3	62.1	7.7	0.0
	4	98.2	0.8	1.5
	5	98.4	0.7	7.8
	6	98.6	0.9	33.4
	7	99.0	0.2	20.4
	8	98.4	1.7	37.5
	9	98.2	0.6	41.5
	12	96.8	2.1	13.2
2854	2	0.1	1.5	0.0
	3	93.7	14.4	0.0
	4	98.6	27.6	1.0
	5	99.0	35.0	1.8
	6	98.0	25.5	0.6
	7	99.9	31.6	0.2
	8	99.9	37.8	0.6
	9	98.2	36.5	1.3
	12	96.4	55.2	2.7

*RIA values of >50% inhibition are positive.
†RIA signal/noise ratio values >3.1 are positive.
‡Solid-phase RIA values >3.1 are positive.

Table 4. Serological profile of woodchucks infected with WHV7R

Woodchuck	Months postinfection	Anti-WHcAg*	WHsAg†	Anti-WHsAg‡
3079	2	2.4	3.3	1.3
	3	79.5	51.4	0.6
	4	98.6	47.7	1.6
	5	98.6	28.5	1.0
	6	99.2	23.6	1.3
	7	98.3	37.3	1.6
	8	99.1	41.3	0.8
	9	98.3	19.7	0.8
	12	98.8	21.5	1.5
3093	2	6.4	14.6	1.6
	3	75.0	26.4	1.2
	4	97.7	0.8	7.2
	5	98.6	1.6	9.6
	6	98.0	0.9	7.0
	7	98.2	1.0	7.1
	8	97.5	0.7	19.6
	9	98.2	0.8	11.1
	12	97.8	0.8	14.0

*RIA values of >50% inhibition are positive.
†RIA signal/noise ratio values >3.1 are positive.
‡Solid-phase RIA values >3.1 are positive.

In the present study, our goal was to determine the degree of variability among WHV genomes isolated from an infected woodchuck. We found evidence for variation among supercoiled genomes from a single, infected woodchuck. Also, we found that two of three recombinants were incapable of independent replication after transfection of neonatal woodchucks. Recombinant pWHV7-3 had a 73-base-pair deletion within the X and precore gene region that excised one of the short direct repeats essential for virus replication. Recombinant pWHV7-9 had only two changes relative to the infectious recombinant pWHV7-11. One change was a silent mutation in the polymerase gene, while the other changed an invariant histidine residue to a proline residue in the core gene. We hypothesize that defective mutants accumulate over time due to complementation by wild-type virus. Although, eventually, the noninfectious mutants may outnumber wild-type virus, we find that the ratio of defective to infectious virus does not appear to exceed a ratio of 10:1 in the serum (B.E.K. and J.L.G., unpublished data). Thus, although it is possible that the majority of circulating virions contain defective genomes, the level of such mutants does not appear to reach the extremes found for defective interfering particles (28).

Although the changes observed in the virus genomes that we sequenced could be the result of artifacts that arose during molecular cloning, we believe they represent the natural variation found among virus genomes in infected woodchucks. This is likely to be the case for several reasons. First, the supercoiled molecules that we cloned are completely double-stranded DNA molecules, and it is unlikely that any change would occur during isolation and cloning of the virus DNA. We avoided cloning virus genomes from serum particles since they contain extensive regions of single-stranded DNA that must be repaired by *in vitro* polymerase reactions that could be a source of error. Second, it is unlikely the mutations arose during propagation of the recombinant plasmid because the bacterial polymerase possesses a proofread-

ing function that accounts for replication of bacterial DNA with extremely high fidelity. However, since it was possible that the WHV genome contained nucleotide sequences that were hot spots for mutation during propagation in *E. coli*, we performed an experiment to address this issue. We found that progeny WHV plasmids that were passaged in bacteria for >250 cell divisions did not develop any nucleotide changes compared to the parental plasmid DNA. We conclude that it is unlikely that there are hot spots of mutation in the WHV genome during propagation as part of a plasmid in *E. coli* and that it is likely that the variations among recombinant WHV supercoiled molecules we have described reflect natural sequence heterogeneity found among virus genomes in infected animals.

It is also unlikely that the virus genome could change substantially in the host during the limited time necessary (i.e., ≤5–6 months) to establish a persistent virus infection. In a previous experiment (29), we determined that the mutation rate of the WHV genome in a persistently infected woodchuck was $\leq 2 \times 10^{-4}$ nucleotide substitutions per genome site per year, which is less than one change per genome per year of continuous replication of the virus. We also found no evidence for a favored site of nucleotide substitution on the WHV genome in that study and conclude that it is unlikely that a significant number of WHV mutants arise within the relatively brief interval necessary to establish persistent WHV infection.

Many studies on viral infection begin with a plaque-purified virus inoculum, which, when generated by passage in culture with a low multiplicity of infection, represents virus with a uniform nucleotide sequence. However, plaque purification of hepadnaviruses is not possible at this time. Therefore, we used a different approach to obtain virus with a uniform nucleotide sequence by transfecting the liver of neonatal woodchucks with an infectious clone of WHV and using the virus produced to make a serum pool. When the rates of persistent WHV infection of the original virus pool (i.e., the WHV7 pool) and the recombinant-derived virus pool (i.e., the WHV7R pool) were compared, we found that 65% and 80% of the woodchucks, respectively, became chronic carriers. In a parallel experiment, using a second recombinant virus pool derived from woodchucks transfected with WHV8 DNA, we found a chronic carrier rate of 70%. Thus, inoculation of susceptible woodchucks with an infectious serum pool that includes defective WHV results in a rate of chronic virus infection similar to that of woodchucks inoculated with an infectious serum pool containing only a single wild-type virus strain. This suggests that defective virus is not essential for the establishment of persistent hepadnavirus infection.

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