Mutant woodchuck hepatitis virus genomes from virions resemble rearranged hepadnaviral integrants in hepatocellular carcinoma

(topoisomerase I/hepatitis B virus)

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ABSTRACT   Although hepadnaviruses are implicated in the etiology of hepatocellular carcinoma, the pathogenic mechanisms involved remain uncertain. Clonally propagated integrations of hepadnaviral DNA into cellular DNA can be demonstrated in most virally induced hepatocellular carcinomas. Integration occurs at random sites in cellular DNA, but the highly preferred sites in viral DNA are adjacent to the directly repeated sequence DR1, less often DR2, or in the cohesive overlap region. Integrants invariably contain simple deletions or complex rearrangements that have been thought to occur after integration. We report here the detection of mutant woodchuck hepatitis virus (WHV) genomes cloned from virions in serum that are strikingly similar to the rearranged hepadnaviral genomes found previously as integrated sequences in cellular DNA. Of 102 cloned genomes studied, 2 had large inverted duplications, 1 a 219-nucleotide direct duplication, and 1 a 219-nucleotide deletion. Virus–virus DNA junctions occurred either adjacent to DR1 or DR2 or in the cohesive overlap region at preferred topoisomerase I cleavage sites. Since these sites are located in the single-stranded regions of the genome, cleavage by topoisomerase I would produce linear molecules that would be expected to be highly recombinogenic since this enzyme, possessing nicking and ligating activities, would remain covalently attached. Sucrose density gradient centrifugation coupled with polymerase chain reaction studies confirmed that the mutant WHV DNA forms resided in virions and did not represent free viral DNA released from infected cells or were unlikely to be an artifact of the cloning process. Thus, the finding in virions of mutant WHV DNA similar to WHV DNA integrated into cellular DNA suggests that the processes of mutation and integration are linked in some instances. Furthermore, the mutant genomes that are preferentially integrated into cellular DNA may have an etiologic role in hepatocarcinogenesis.

Hepadnaviruses are small enveloped viruses with a partially double-stranded DNA genome. Included in the family are hepatitis B virus (HBV), woodchuck hepatitis virus (WHV), and ground squirrel hepatitis virus, all of which are associated with development of hepatocellular carcinoma (HCC) in their respective hosts, and duck hepatitis B virus, which has not been closely linked with HCC. HCCs contain integrated viral DNA with deletions or direct or inverted duplications. Preferred virus–virus and virus–cell DNA junctions are localized to a defined region of the virus genome between DR1 and DR2 containing the cohesive overlap region (COR) (1–7). Viral integrants are found in most, but not all, HCCs examined, and their existence provides support for the hypothesis that integration plays a role in oncogenesis.

WHV infection of woodchucks (*Marmota monax*) has proven to be an extremely useful model for studying the biology of infection with hepadnaviruses, especially the pathogenic mechanisms responsible for the development of HCC (5). The goal of this study was to characterize the virus genomes from the serum of a woodchuck that was a chronic carrier of WHV and had HCC. In this analysis we found evidence for the presence of hepadnaviral mutants that resemble the rearranged genomes found previously as integrated sequences in cellular DNA.

MATERIALS AND METHODS

Animals. Woodchucks were trapped in central New York state (Tompkins County), an area in which the prevalence of WHV infection is low (<0.1%), and tested for serologic markers of WHV infection. Woodchucks WC1993, WC2107, WC2153, WC2328, and WC2761 were positive for WHV surface antigen (WHsAg) at the time they were trapped. Woodchucks WC1993, WC2107, and WC2761 were subsequently shown to have persistent WHV infection; all three developed HCC after being housed in the laboratory for ≈1 year.

Molecular Cloning. Virus particles were purified from 10 ml (≈10^8 virus genomes per ml) of serum from WC2761 by sucrose gradient centrifugation, and the plus strand of the viral DNA was elongated using the endogenous polymerase reaction. Viral DNA was isolated by digestion with proteinase K in the presence of sodium dodecyl sulfate, followed by phenol/chloroform extraction and ethanol precipitation. Next, the DNA was made fully double-stranded by treatment with avian myeloblastosis virus reverse transcriptase. WHV DNA was then digested with restriction endonuclease HindIII, ligated into the HindIII site of pUC18, and cloned in *Escherichia coli* (strain JM 109). WHV DNA-containing recombinants were identified by colony filter hybridization. DNA was extracted from each clone, digested with HindIII, and fractionated by agarose gel electrophoresis to characterize the WHV genome inserts. A total of 102 clones was selected for analysis: 98 clones possessed WHV DNA of unit length, 2 clones had genomes larger than genome size, and 2 clones had genomes that were smaller than genome size.

DNA Sequencing and Computer Analysis. The complete nucleotide sequence of one of the unit length clones (clone 10) was determined by the dideoxy method and has been deposited with the EMBL/GenBank libraries under accession no. M90520 (8). Computer analysis of nucleotide and predicted amino acid sequences was conducted using the GenBank Online Service.

Amplification of WHV DNA from Woodchuck Serum by PCR. WHV DNA was amplified in a "nested" PCR assay using primer pairs specific for the mutated regions of the genome as described (9). The PCR products were fractionated by 2% agarose gel electrophoresis and isolated by electroelution, and the DNA fragments were either sequenced directly by a modified dideoxy chain-termination method in the presence of dimethyl sulfoxide (10) or cloned into pUC18 and the plasmid DNA was sequenced.

Sucrose Gradient Centrifugation of Woodchuck Serum from WC2761. Virions in the serum of WC2761 were banded in a sucrose gradient for 18 hr at 150,000 × *g* at 4°C. The gradients were formed as follows: 1 ml of 66% sucrose (wt/wt) was pipeted into the bottom of the tube, followed by sequential addition of 2 ml each of 55%, 40%, 30%, 20%, and 10% sucrose (wt/wt), and overlaid with 1 ml of the serum sample. Two control gradients overlaid with 1 ml of negative woodchuck serum, or phosphate-buffered saline, were run as controls to obtain density measurements by refractometry. After centrifugation, 0.5-ml fractions were collected from the bottom of each tube. Each fraction was stabilized by adding 50 μl of 1% bovine serum albumin. Two microliters of each fraction was tested for the presence, as well as the titer, of WHsAg as described (11). Fifty microliters of each fraction was tested for WHV DNA by slot-blot hybridization. Selected fractions were tested in parallel with a negative control sample containing unfractionated serum from WC2550, a woodchuck that was negative for all markers of WHV infection, by extraction of DNA from 10 μl and examination by nested PCR amplification using primer pairs bracketing the mutated region(s) of the WHV genome. Unfractionated serum from WC2761 served as a positive control.

RESULTS

Cloning of WHV DNA from the Serum of a Chronically Infected Woodchuck. We performed a comprehensive analysis of WHV genomes recovered from virions in the serum of WC2761. Virus particles from serum were isolated by centrifugation and the plus strand of viral DNA was elongated by the endogenous polymerase reaction. After purification, the DNA was made fully double-stranded by avian myeloblastosis virus reverse transcriptase and cloned into the *Hind*III site of pUC18 (see *Materials and Methods*). WHV recombinants were identified by colony filter hybridization. DNA was extracted from each recombinant, digested with *Hind*III, and fractionated by agarose gel electrophoresis to determine the length of the WHV insert.

Analysis of WHV Genomes. Of the 102 recombinants, 98 were of genome length, 2 clones were shorter than genome length, and 2 were longer. Both strands of DNA from one representative clone of genome length (i.e., 3308 bp) were sequenced by Sanger's dideoxynucleotide method to characterize the genome (8). Compared to the previously published sequences of WHV, the sequence of our recombinant genome was most similar to that reported by Galibert *et al.* (12). The four mutant genomes were characterized by mapping with restriction endonucleases and by DNA sequencing (Figs. 1 and 2). Mutant clone 18 possessed a 219-nt in-frame deletion between map positions 1717 and 1935, resulting in truncation of the C, P, and X genes (Fig. 1A). Mutant clone 35 had a genome length of 2285 bp and possessed an inverted duplication interrupted by a 235-nt deletion (Fig. 1B). This rearrangement resulted in loss of the X gene and truncation of the C, S, and P genes. Mutant clone 72 had a 219-bp
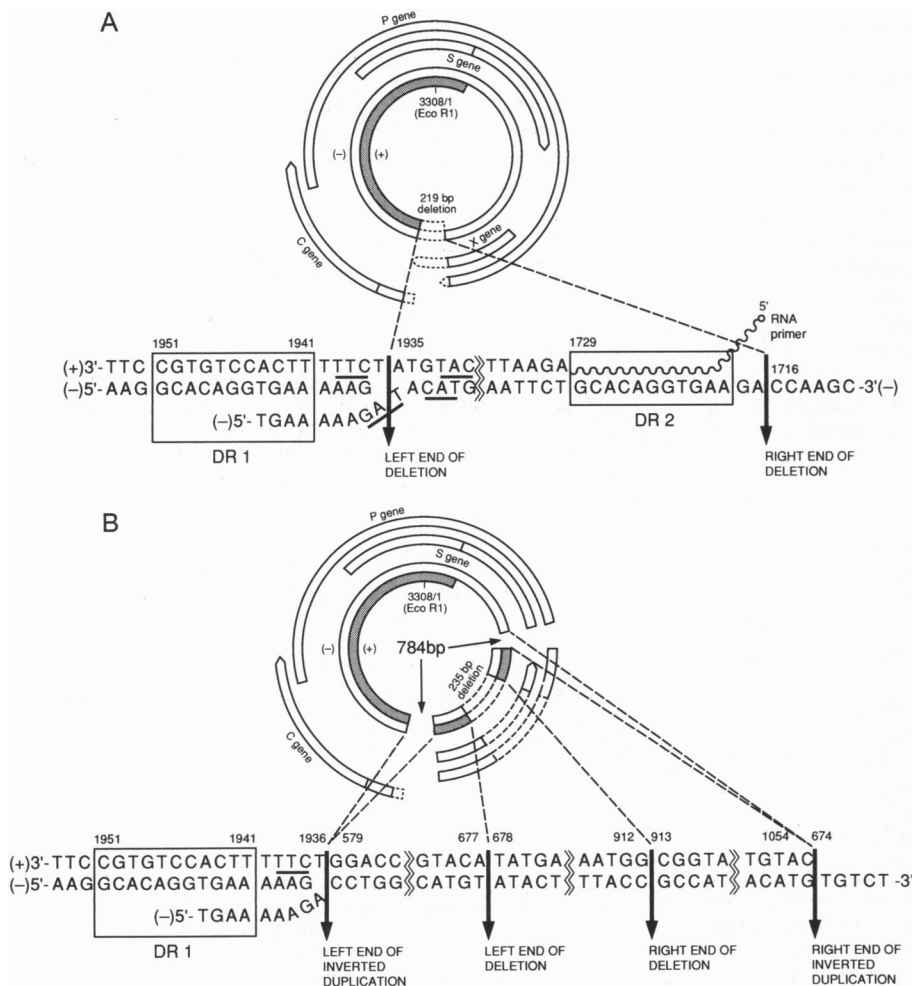
FIG. 1. Mutant WHV genomes that are shorter than genome length in size. (*A*) Clone 18 (3089 bp) with a 219-nt in-frame deletion between map positions 1717 (adjacent to DR2) and 1935 (adjacent to DR1). This would cause severe truncation of the X gene, loss of enhancer II, truncation of the extreme 3' end of the polymerase gene, and absence of the extreme 5' end of the precore gene with loss of the initiation codons for this gene. The site of initiation of minus strand synthesis and the RNA packaging signal are present. The first 4 nt at the right end of the deletion (TCTT) are the same as the first 4 nt in the sequence immediately to the left of the deletion. Map unit designations here and Fig. 2 are those from Cohen *et al.* (13). The viral core (C), surface (S), polymerase (P), and X genes are shown. The precore gene lies immediately to the 5' end of the core gene. Topoisomerase cleavage motifs are underlined. DR1 and DR2 are boxed. (*B*) Clone 35, exhibiting deletions and a marked degree of DNA rearrangement. The genome length was 2285 nt (length of wild-type genome 3308) with an inverted duplication (one virus–virus DNA junction at 674:1054, the other at 1935:579 with 1935 adjacent to DR1) interrupted by a 235-nt deletion (from nt 678 to nt 912). An additional 784 nt were deleted at the left and/or right end of the inverted duplication. This rearrangement resulted in complete loss of the X gene and severe truncation of the S and P genes and had the same effect on the precore gene as in clone 18.

in-frame direct duplication of nts 1717–1935 (Fig. 2*A*). Finally, mutant clone 76 had a long inverted duplication and was nearly twice the normal WHV genome length (Fig. 2*B*). All four mutant genomes had an intact RNA packaging signal and were capable of being packaged into nucleocapsids.

PCR Amplification of Virus–Virus Junctions in WHV Mutants. To exclude cloning artifacts as a cause of the DNA rearrangements found in the present study, we used PCR to amplify a unique DNA fragment spanning the virus–virus DNA junctions of mutant clone 18 (with a deletion) from the serum of WC2761. The amplified DNA was sequenced directly (10) and was also cloned into pUC18 and sequenced (13). The results of both experiments were in agreement and the presence of the exact deletion found in mutant clone 18 (spanning bases 1717–1935) was confirmed. Thus, using two techniques (i.e., cloning and PCR amplification) coupled with DNA sequencing, we found evidence for the presence of WHV genomes with a precise deletion in virions from the serum of WC2761.

To show that the virus mutants were not confined to virions from the serum of a single animal, sera from four other woodchucks chronically infected with WHV, two with (WC1993 and WC2107) and two without (WC2153 and WC2328) HCC, also were examined by PCR analysis. A band equal in size to that expected for the virus–virus DNA junction of clone 35 (with an inverted duplication) was amplified from the viral DNA extracted from serum of both woodchucks with HCC and one without evidence of HCC. Similarly, a band equal in size to that expected for the virus–virus DNA junction of clone 18 (with a 219-bp deletion) was identified in the DNA isolated from the serum of one of the two woodchucks with HCC. Direct sequencing of the PCR product from one of the sera showed nucleotide se-

quences consistent with an inverted duplication at base 1935 analogous to the virus–virus junction fragment of mutant clone 35. Thus, the presence of WHV genomes with specific mutations was confirmed in four of five woodchucks with chronic WHV infection.

Sucrose Gradient Banding of Virions from Woodchuck Serum. To confirm that mutant virus genomes were encapsidated in virions in the serum, rather than existing as free DNA forms released from lysed cells, we performed sucrose gradient centrifugation on serum obtained from WC2761 (see *Materials and Methods*). Assays for WHsAg as well as WHV DNA hybridization were performed on each fraction (Fig. 3). WHsAg was found in fractions 7–9 with the peak at fraction 8 (1.18 g/ml). Slot-blot hybridization with a radioactive probe specific for the WHV genome revealed that most WHV DNA was found in fractions 5–9 with the main peak at fraction 6. Representative samples were selected throughout the gradient (i.e., fractions 2, 4, 6, 8, 10, 12, 18, and 24) for further analysis. To avoid detecting minute amounts of wild-type and mutant WHV DNA that could be present throughout the gradient, we used a small aliquot (i.e., 10 μl) of each fraction for DNA extraction and, furthermore, used only 10% of the resulting DNA sample for nested PCR amplification (Fig. 4). Thus, analysis of the DNA from the equivalent of 1 μl of selected gradient fractions revealed that wild-type WHV DNA was present in fractions 6, 8, and 10 and mutant WHV DNA was present in fraction 6 (1.21 g/ml). This finding demonstrates that mutant WHV DNA comigrates in sucrose gradients with intact viral particles, suggesting that this DNA is packaged into virions.

DISCUSSION

The mutant WHV genomes from serum that we have identified bear a striking resemblance to the clonally propagated
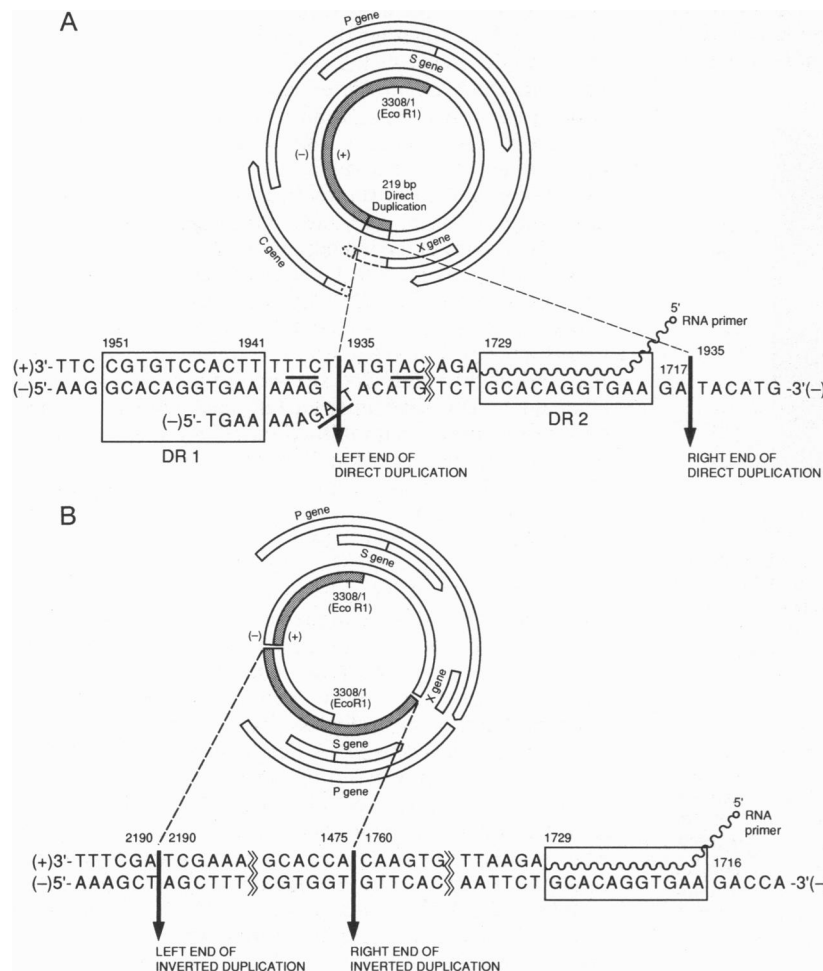
FIG. 2. Mutant WHV genomes that are greater than genome length in size. (*A*) Clone 72 (3527 bp), exhibiting a 219-nt in-frame direct duplication at base 1935, repeating nt 1717–1935 (the direct duplication thus consists of the cohesive end region between DR2 and DR1). The first 4 nt at the right end of the direct duplication (TCTT) are the same as the first 4 nt in the sequence immediately to the left of the direct duplication. The virus core (C), surface (S), polymerase (P), and X genes are shown. Topoisomerase cleavage motifs are underlined. DR1 and DR2 are boxed. (*B*) Clone 76, consisting of almost two complete genomes (genome length 5471 nt). This mutant has a very long inverted duplication with one virus–virus DNA junction between positions 1760 (in the COR near DR2) and 1475 and the other at position 2190:2190.
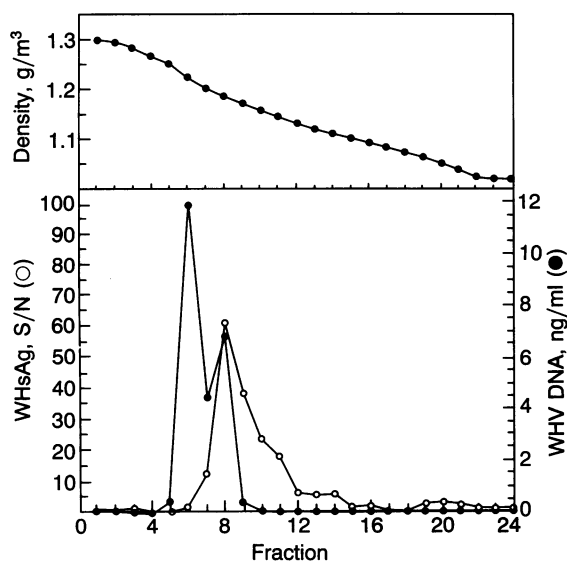
FIG. 3. Sucrose gradient centrifugation of serum from WC2761. Sucrose gradient preparation and centrifugation were performed as described in the text and fractions were collected from the bottom of the tube. (*Upper*) Density profile of an identical gradient prepared in parallel with density presented as g/cm^3. (*Lower*) Fractions were tested for the presence of WHsAg, expressed as the signal-to-noise ratio (S/N), and WHV DNA. The majority of WHsAg occurs as incomplete particulate forms with a lower density than intact virions.

integrants of HBV DNA and WHV DNA in human and woodchuck HCCs, respectively, reported previously (1–7). HCCs from both species contain viral integrants with deletions or direct or inverted duplications resulting in DNA segments that are either shorter or appreciably longer than genome length. Moreover, the preferred sites of both virus–
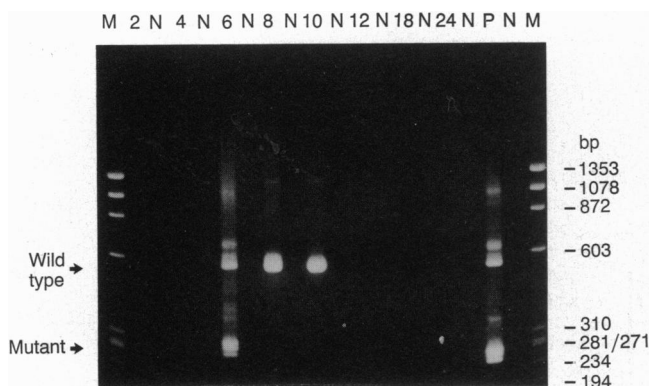

FIG. 4. Agarose gel electrophoresis of PCR-amplified WHV DNA purified from fractions of a sucrose gradient. Representative fractions from the gradient shown in Fig. 3 were paired with negative control samples (unfractionated serum of WC2550, a woodchuck that had no serological evidence of previous or current WHV infection). DNA extraction and PCR amplification were performed with WHV-negative serum samples interspersed among the experimental samples as a test for false positive results caused by contamination. A 2% agarose gel was used to fractionate the amplified DNA sequences. DNA was stained with ethidium bromide and visualized under UV light. Lanes M, molecular weight marker ϕX174 DNA digested with restriction endonuclease *Hae* III. DNA bands are given in base pairs (bp). Lanes 2, 4, 6, 8, 10, 12, 18, and 24 contain the PCR amplification products from DNA isolated from analogous fractions 2, 4, 6, 8, 10, 12, 18, and 24 of the sucrose gradient, respectively. Lane P, PCR amplification products from DNA isolated from the positive control (unfractionated serum from WC2761). Mutant and expected wild-type DNA bands are highlighted with arrows. Lanes N, PCR amplification products from DNA isolated from the negative control (unfractionated serum of WC2550).

Table 1. Analysis of virus–cellular and virus–virus DNA junctions of integrants of HBV DNA in HCC

Integrant	DR1	DR2	COR	Total*
Virus–cellular DNA junction	18	4	5	27
	(42.9)	(9.5)	(11.9)	(64.3)
Virus–virus DNA junction	6	0	2	8
	(14.3)		(4.8)	(19.0)

n = 42. Percentages are in parentheses.
*The DR1, DR2, and cohesive overlap regions occupy only 7% of the HBV genome.

virus DNA junctions in the mutants and of virus–cellular DNA and virus–virus DNA junctions in the integrants are immediately adjacent to DR1 or, less often, DR2 or in the remainder of the COR (Table 1). Judging from the high frequency with which virus–cellular junctions occur at either DR1 or DR2, these loci appear to be important for the integration of hepadnaviral DNA into cellular DNA (2, 6, 14).

To explain the involvement of DR1, DR2, and the COR in the generation of viral mutants and integrants it is important to examine the replication cycle of the hepadnavirus genome. First, partially double-stranded DNA genomes enter the nuclei of infected cells and are converted into fully double-stranded, supercoiled molecules. Pregenomic RNA is transcribed from the supercoiled DNA template and packaged. Minus strand DNA is then synthesized by reverse transcription of the RNA pregenome with DR1 and DR2 playing key roles (15). Synthesis of the minus strand of DNA begins in DR1 by utilizing a protein primer. DR1 RNA from the terminal repeat of the pregenome is transposed to base pair with DR2 on the minus strand of DNA, thereby serving as a primer for synthesis of the plus strand of DNA. Since plus strand DNA synthesis is incomplete, the resulting molecule is a circle held together by hydrogen bonding of nucleotides in the COR. Because the replication of both strands of the genomic DNA occurs after packaging, they are not covalently joined until after reinfection of the same or another hepatocyte (16). There is, therefore, a nick adjacent to DR1 in the minus strand and a single-stranded region 5' to DR2 in the plus strand until reinfection, at which time uncoating, elongation of the plus strand, and ligation occur.

The structure of the hepadnavirus genome is relevant to the action of topoisomerase I, a cellular enzyme that alters the superhelical state of DNA by nicking–closing reactions on single- and double-stranded DNA (17). Although there are topoisomerase I cleavage sites throughout the WHV genome, there are highly preferred sites in the single-stranded region adjacent to DR1 and in the COR near DR2 (14). Nicking–closing reactions on double-stranded genome regions would not result in conformational change. Cleavage at the single-stranded DNA regions, however, would result in linearization of the hepadnaviral genome, with topoisomerase I remaining covalently bound to the 3' phosphate at the nick site (18, 19), producing a highly recombinogenic DNA molecule.

There are several lines of evidence suggesting that topoisomerase I could be involved in the generation of the virus mutants that we identified in serum. (*i*) In an *in vitro* assay topoisomerase I has been shown to mediate linkage of WHV DNA to both 5' OH acceptor ends of heterologous DNA fragments and to internal sites of linear double-stranded cellular DNA (19). (*ii*) Analysis of virus–virus DNA junctions from a group of WHV mutants, termed "novel forms," identified in hepatocyte nuclei of chronically infected animals without HCC (20) revealed that all contained one or more topoisomerase I cleavage motifs (P. Schirmacher and C. E. Rogler, personal communication). (*iii*) Analysis of hepadnaviral recombination sites from integrants found in HCC DNA revealed that >90% of the viral recombination sites contained preferred topoisomerase I cleavage motifs (P. Schirmacher and C. E. Rogler, personal communica-

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tion). (*iv*) Topoisomerase I cleavage sites occur at, or near, all virus–virus DNA junctions in the four mutants we have characterized. Thus, it is likely that topoisomerase I is involved in the formation of mutant genomes.

The resemblance between the mutant WHV genomes that we cloned from the serum of an infected woodchuck and the novel forms discovered by Rogler and coworkers raised the unlikely possibility that we had cloned unpackaged DNA that was released from infected cells by lysis. Although the first step in our cloning procedure (i.e., purification of virions by pelleting through a sucrose gradient) should have eliminated any free DNA that survived the naturally occurring nucleases found in serum, we performed several experiments to demonstrate that the mutant genomes resided in virions and did not represent unpackaged WHV DNA released from the nuclei of infected cells. The results of these experiments substantiated the hypothesis that the mutant WHV DNA was encapsidated in virions in serum.

Our results suggest that mutation of the hepadnaviral genome occurs either during or after conversion of the circular virus genome to a linear conformation by topoisomerase cleavage of single-stranded regions. Direct and inverted duplications could occur by recombination between hepadnaviral genomes. Deletions could occur as the result of homologous recombination between short stretches of identical nucleotides within a viral genome. Pursuant to the latter hypothesis we observed a 4-nt homology at sites of recombination in two of the mutants (Figs. 1 and 2). Similar findings were also observed by Wang and Rogler (19) for *in vitro* topoisomerase I-generated integrations of WHV DNA. In addition, it has been shown that vaccinia virus topoisomerase I can mediate analogous rearrangements (21).

Topoisomerase I mutagenesis of hepadnaviral DNA is most likely to occur in the nucleus of infected cells prior to conversion of partially double-stranded DNA to supercoiled DNA. Mutated genomes would be converted into supercoiled DNA templates and the rearranged genomes would be propagated in the normal replicative pathway. However, it is also possible that topoisomerase is encapsidated and that mutagenesis occurs in the nucleocapsid during conversion of the viral RNA pregenome into the partially double-stranded DNA form. In support of the latter notion, two of our mutants (clones 18 and 35) lack DR2 and would be unlikely to exist as functional templates for genome replication. Although it is possible that a closely related sequence substitutes for the cis-acting function of DR2 and that replication proceeds along the normal pathway, it is also feasible that deletion mutations are generated *de novo* at a relatively high rate due to recombination at preferred cleavage sites.

It should be noted that we cannot completely rule out the possibility that these mutants were formed during the cloning process or as a consequence of PCR amplification. One would expect that the COR of the hepadnaviral genome, with a protein attached to the 5′ end of the minus strand and an RNA primer attached to the 5′ end of the plus strand, would be difficult to clone or amplify accurately. However, to our knowledge, there are no previous reports documenting unusual difficulty in this regard. Considering that it is unlikely that an identical 219-bp deletion would occur in both processes, that all virus–virus junctions occur at topoisomerase I cleavage sites, and that mutants are found in some, but not all, animals studied, we conclude that mutants are generated at a low frequency during the course of infection.

The similarity between the mutants we have described and integrants reported in HCC suggests that mutants may be preferentially (perhaps exclusively) integrated into cellular DNA. Topoisomerase I is involved in replication of cellular DNA and its concentration is increased in cells during cell division (22, 23). In this way, the increased hepatocyte turnover rate associated with viral hepatitis may promote

integration of viral DNA by the action of topoisomerase I covalently bound to hepadnaviral DNA. Thus, the process of mutation of the hepadnaviral genome may be linked, in some cases, to integration into cellular DNA. In fact, complex rearrangements of integrated viral sequences have been found in cells transformed by simian virus 40 (SV40) (24, 25) and adenovirus 2 (26), suggesting that preferential integration of mutant viruses into cellular DNA may be a general phenomenon. Moreover, topoisomerase I cleaves SV40 DNA *in vitro* in the same positions that viral excision and rearrangement occur (27, 28). Mutant genomes such as those reported here may not have been detected previously because they constitute a small proportion ($\approx$4%) of the genomes present in the virions from serum.

It has been assumed that rearrangements of integrated hepadnaviral DNA occur after integration into host DNA. The finding, in virions from the serum, of mutant genomes resembling viral integrants suggests that alteration of the virus genome may precede integration, which is believed to have an etiologic role in hepatocarcinogenesis.

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