Out-of-Frame versus In-Frame Core Internal Deletion Variants of Human and Woodchuck Hepatitis B Viruses

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Human hepatitis B virus (HBV) variants containing in-frame core internal deletion (CID) have been demonstrated to contain all the functional features of defective interfering (DI) particles (Yuan, T. T.-T., M.-H. Lin, D. S. Chen, and C. Shih, 1998, J. Virol. 72, 578–584). Here, we report that out-of-frame HBV CID variants exhibit defective interfering property similar to in-frame CID variants characterized previously. This result raises the possibility that it may be the deleted pregenomic RNA product, rather than the deleted core protein product, that is responsible for interference. Furthermore, a genomic deletion elsewhere does not cause interference since preS2 deletion variants exhibit no influence on wild-type HBV replication. Consistent with the natural occurrence of HBV CID variants, we recently identified CID variants of woodchuck hepatitis virus (WHV) in natural infection. However, unlike HBV CID variants, functional characterization of WHV CID variants using a human hepatoma cell line has not revealed any interference in tissue culture. In summary, defective interference is a general phenomenon for both in-frame and out-of-frame HBV CID variants.

Key Words: HBV; hepatitis B virus; WHV; woodchuck hepatitis B virus; CID; core internal deletion; DI particle; defective interfering particle; variants; capsid.

INTRODUCTION

Hepatitis B virus (HBV) is a hepatotropic infectious agent in humans. Worldwide, there are at least 300 million chronic carriers of HBV. Chronic active hepatitis associated with HBV infection often leads to the development of cirrhosis and hepatocellular carcinoma (HCC) (Chen, 1993; Shih et al., 1996; Butel, 2000). In contrast, self-limited acute hepatitis B is rarely associated with liver cancer. The mechanisms of pathogenesis and chronicity of HBV infection remain to be elucidated.

Recently, naturally occurring variants containing core antigen internal deletions (CID) have been found in chronic HBV carriers (Okamoto et al., 1987; Wakita et al., 1991; Ackrill et al., 1993; Akarca and Lok, 1995; Uchida et al., 1994; Gunther et al., 1995) and in patients with hepatocellular carcinoma (HCC) (Hosono et al., 1995). In Taiwanese children, CID variants were found to be more associated with horizontal transmission (Ni et al., 2000). Interestingly, CID variants have rarely, if ever, been found in acute hepatitis patients (Ehata et al., 1993; Aye et al., 1994). The involvement of CID variants in HBV-related liver disease remains unclear. Recent studies indicate that CID variants bearing an in-frame core internal deletion are by themselves replication defective, but can be rescued by providing core protein in trans (Okamoto et al., 1993; Yuan et al., 1998a; Gunther et al., 2000). Furthermore, such an in-frame CID variant can replicate preferentially at the expense of the wild-type helper virus (Yuan et al., 1998b), a phenomenon reminiscent of defective interfering (DI) particles (von Magnus, 1947; Huang and Baltimore, 1970; Holland, 1987). In addition to in-frame CID variants, out-of-frame HBV CID variants have also been reported previously (Wakita et al., 1991; Okamoto et al., 1993; Fiordalisi et al., 1994; Marinos et al., 1996). To date, it remains unclear if the out-of-frame (3n+1 and 3n+2) CID variants also behave like DI particles.

Woodchuck hepatitis virus (WHV) is closely related to human hepatitis B virus in many aspects, including their respective morphology, genome structure, replication strategy, epidemiology, and pathobiology. WHV shares about 65% nucleotide sequence homology with HBV (Koda, 1985). Similar to HBV, WHV can establish chronic infection in woodchucks as natural hosts, and this leads to the development of HCC (Popper et al., 1987; Tennant and Gerin, 2001). In woodchucks from Pennsylvania and North Carolina, Li et al. (1996) reported the existence of WHV e antigen-negative variants. Most recently, Botta et al. (2000) reported the existence of WHV CID variants in natural infection. It is possible that other kinds of variants can be found in the WHV/woodchuck system (Ou, 1997).
Here, we describe the functional characterizations of WHV CID variants in tissue culture. Furthermore, we compared the replication behavior between in-frame vs out-of-frame CID variants from humans and woodchucks. Similar to the in-frame HBV CID variants (Yuan et al., 1998a,b), our results demonstrated the DI-like properties of out-of-frame HBV CID variants.

RESULTS

Rescue of HBV out-of-frame CID variants via transcomplementation with a wild-type core antigen

Previously, it has been demonstrated that an HBV in-frame CID variant can be rescued by providing a wild-type core protein in trans (Okamoto et al., 1993; Yuan et al., 1998a; Gunther et al., 2000). It remains unclear if the out-of-frame HBV CID variants can be rescued experimentally in a similar manner. To this end, we constructed two out-of-frame HBV mutants by introducing a deletion of one or two nucleotides (boxed T or TC, Fig. 1A) at a core gene region upstream from the in-frame deletion junction in the plasmid pDEL85 (Yuan et al., 1998a,b). As shown in Fig. 1B, both \( 3n+1 \) and \( 3n+2 \) CID variants are replication defective by themselves. However, when cotransfected with pSVC which provides a wild-type HBV core protein in trans, both \( 3n+1 \) and \( 3n+2 \) deletion variants can be successfully rescued.

Interference and enrichment property of HBV out-of-frame CID variants

To compare the interference activity among in-frame and out-of-frame CID variants (if any), we used the previously established “1-filter-3-probe assay” (Yuan et al., 1998b). Equal amounts of plasmids expressing wild-type and CID variants were cotransfected into the HuH7 cell line. As shown in Fig. 1C, no significant difference in the total HBV DNA synthesis was observed when full-length HBV probe (first probe) was used in the Southern blot analysis. However, when a wild-type specific probe (second probe) was used for the same filter instead, significant reduction of wild-type DNA replication (i.e., interference) was apparent in all three kinds of CID variants (lanes 2, 3, and 4, Fig. 1D). The decrease of the wild-type population appears to be compensated by the simultaneous increase of the mutant-specific HBV DNA population (third probe) (i.e., enrichment) (lanes 2, 3, and 4, Fig. 1E). An average of approximately 3-fold enrichment and 5- to 10-fold interference was observed in both in-frame and out-of-frame HBV CID variants (the method of calculation was as detailed in Yuan et al., 1998b). These results of quantitative Southern blot analysis indicate that HBV CID variants can interfere with the wild-type virus replication in a manner independent from their core open reading frames.

Internal deletions of WHV core antigens

As shown in Fig. 2, we have identified three serum samples (woodchuck Nos. 107, 111, and 362) that contained detectable WHV CID variants. The WHV CID variants were always accompanied by WHV helper virus containing an apparent full-length core gene. The relative abundance between full-length WHV and the CID variants varied between samples, ranging from approximately 1:1 to 4:1 (Fig. 2A). Similar to the case of HBV, both in-frame and out-of-frame deletions were present in the WHV CID variant population in woodchuck No. 107 (Fig. 2B). The size of the deletions varied from 29 to 104 amino acids. The deletions were often located within the central portion of the WHV core antigen (between amino acids 80 and 131). These hotspot deletion regions were mapped outside the arginine-rich, nucleic acid-binding domain near the carboxyl terminus of the core gene (Gallina et al., 1989; Birbaum and Nassal, 1990; Haton et al., 1992). Interestingly, the deletion coincides with a reported immunodominant T cell epitope of core antigen (Milich et al., 1987; Jung et al., 1995; Tsai et al., 1996; Menne et al., 1997). Except for woodchuck No. 362, none of the deletions extended into the adjacent polymerase open reading frame. In the sample from woodchuck No. 362, the deletion extended to the first 11 amino acids of the polymerase (Fig. 2B). The population of CID variants in woodchuck No. 107 appeared to be more heterogeneous than those from woodchuck Nos. 111 and 362 (Fig. 2B).

In summary, all of the aforementioned features of WHV CID variants are similar to the reported HBV CID variants in human patients over the past decade (Okamoto et al., 1987; Wakita et al., 1991; Ackrill et al., 1993; Okamoto et al., 1993; Uchida et al., 1994; Fiordalisi et al., 1994; Akarca and Lok, 1995; Hosono et al., 1995; Marinos et al., 1996). Of note, \( 3n+2 \) out-of-frame WHV CID variants (62%) are more frequent than \( 3n+1 \) out-of-frame (2%) and in-frame (36%) CID variants in woodchuck No. 107 (data not shown). In the case of HBV, CID variants could exist in the same individual as a relatively homogeneous population. Alternatively, they could also exist as a more heterogeneous mixture with variable sizes and locations of internal deletions. In the latter case, the relative abundance between the in-frame and out-of-frame HBV CID variants have not been quantitatively compared (Wakita et al., 1991; Ackrill et al., 1993; Okamoto et al., 1993; Fiordalisi et al., 1994; Akarca and Lok, 1995; Marinos et al., 1996).

Rescue of WHV CID variants via transcomplementation with a wild-type WHV core antigen

To ask if the reading frames of the deleted core gene influence any selective advantage for WHV CID variants, we constructed WHV CID variants containing core internal deletions at all three possible reading frames: \( 3n \),
FIG. 1. (A) Constructions of HBV out-of-frame CID variants. Boxed nucleotides represent deleted sequences to create out-of-frame mutants. The bold italic nucleotides TA, which coincide with the ATG initiation codon of the polymerase gene, represent the deletion junction in pDEL85 core gene. (B) Transcomplementation of in-frame and out-of-frame HBV CID variants. Ten micrograms of plasmids containing HBV CID variants were transfected into Huh7 cells with or without 10 μg of plasmid pSVC expressing wild-type HBV core protein. Viral DNA associated with intracellular core particles were subjected to Southern blot analysis using 32P-labeled 3.2-kb full-length HBV DNA as a probe. (C, D, and E) Demonstration of defective interference of in-frame and out-of-frame HBV CID variants with the wild-type HBV using a 1-filter-3-probe approach (see text; Yuan et al., 1998b). Ten micrograms of plasmid pWT were transfected into Huh7 cells with or without 10 μg of plasmid containing HBV CID variants. Intracellular core particle-associated viral DNA was isolated 5 days after transfection and analyzed by Southern blot hybridization using 32P-labeled 3.2-kb full-length HBV DNA (see Materials and Methods) as a probe (Fig. 1C). The filter used in the Fig. 1C was washed to remove the full-length probe and rehybridized with 32P-labeled wild-type specific DNA (see Materials and Methods) (Fig. 1D). Finally, the same filter was probed with 32P-labeled HBV CID mutant-specific DNA (Fig. 1E) after removing the wild-type specific probe. Lane 1: pWT; Lane 2: pWT plus pDEL85; Lane 3: pWT plus pDEL85-(3n+1); Lane 4: pWT plus pDEL85-(3n+2). The faint "signal" in Lane 1, Fig. 1E is caused by non-specific cross-hybridization between the mutant-specific probe and the wild-type HBV DNA.
3n+1, and 3n+2. As shown in Fig. 3A, we replaced the HindIII–BglII fragment of pCMW82 with mutant DNA fragments containing naturally occurring in-frame and out-of-frame WHV core internal deletions. None of the WHV CID variants are able to replicate by themselves (data not shown). By analogy to HBV CID variants, it is very likely that the replication defect of WHV CID variants results from a highly unstable CID-specific core protein (Yuan et al., 1998a).

For the complementation assay, we cloned the wild-type WHV core gene from pCMW82 into a CMV vector (see Materials and Methods). Expression of WHcAg under the CMV immediate early promoter from this vector was confirmed by transfection and immunoblot analysis using an anti-HBcAg polyclonal antibody. Because of the strong amino acid sequence homology between HBcAg and WHcAg, we observed strong cross-reactivity between HBcAg antibody and WHcAg produced from this pCMV-WHcAg expression vector (data not shown). As shown in Fig. 3B, all of the WHV CID variants, in-frame or out-of-frame, can be efficiently rescued using a CMV vector expressing wild-type WHcAg. This explains why WHV CID variants are always accompanied by the wild type in natural infection (Fig. 2A). Despite the rescuability of WHV CID variants, we observed no apparent interference in the WHV system using a human hepatoma cell line Huh7 (Fig. 3C), while the positive control of HBV CID variant (DEL85) demonstrated significant interference (Fig. 3D).

**DISCUSSION**

Out-of-frame CID variants fulfill all the functional definitions of DI-like particles

The definition of DI particles includes the following: replication defect in the absence of helper viruses, res-
cuability in the presence of helper viruses, interference with the replication of their helper viruses, and enrichment of DI variants at the expense of helper viruses (Huang and Baltimore, 1970). All four criteria essential for defining a DI particle are based on functional, rather than structural, features. Therefore, HBV out-of-frame CID variants appear to have similar defective interfering capabilities as the in-frame variants (Fig. 1). While deletions of viral genomes are neither necessary nor sufficient for defining a DI particle, many DI particles in other viruses are often associated with deletions (Holland, 1987; Dimmock, 1996). Our characterization of HBV variants containing an internal deletion in preS2 demonstrated that genomic deletion alone is not necessarily a structural signature of DI-like particles (data not shown). In this case of WHV CID variants, we have no evidence of their interference and enrichment property (Fig. 3C) to define them as DI particles, despite their replication defect and rescuability by helper viruses (Fig. 3B).

**The lack of interference and enrichment phenotype of WHV CID variants**

Figure 3 illustrates our effort to extend the interference and enrichment phenomenon from the HBV CID variants to the WHV system in culture. The lack of DI-like phenotype in WHV CID variants could be due to a number of possibilities: for example, species barrier between WHV and the human Huh7 cell line used in the interference assay, or perhaps CMV promoter, is too strong to display properly a DI-like phenotype. Additional mutations outside the *HindIII–BglII* fragment (Fig. 3A), other than the CID mutation, may be required for the interfering behavior in the woodchuck system. Factors other than these...
hypothetical examples may explain the different results for WHV mutants. Further investigation is warranted.

Botta et al. (2000) reported the finding of WHV CID variants in naturally infected woodchucks. Some of their WHV CID variants are similar, in size and location of deletions, to what we have identified in Fig. 2. The biological significance of WHV in-frame CID variants was studied recently by experimental infection of four naive woodchucks (Lu et al., 2001). The results suggest that WHV CID variants did not contribute significantly to the course of acute self-limiting WHV infection. The absence of detectable DI-like phenotype of WHV CID variants in tissue culture (Fig. 3) is consistent with the lack of a significant role of CID variants in acute self-limiting WHV infection.

Evolution and selective pressure for or against in-frame or out-of-frame core internal deletion

Both in-frame and out-of-frame deletions of HBV CID variants have been observed in patients (Okamoto et al., 1987; Wakita et al., 1991; Ackrill et al., 1993; Okamoto et al., 1993; Uchida et al., 1994; Fiordalisi et al., 1994; Akarca and Lok, 1995; Hosono et al., 1995; Marinos et al., 1996). In woodchuck No. 111, the WHV 3n+1 out-of-frame CID variant is the predominant population, while in woodchuck No. 107, the 3n+2 WHV CID variant is the predominant population (Fig. 2). Among the 53 randomly picked bacterial clones from this animal, only one of 53 clones has a 3n+1 CID deletion, resulting in the gene fusion between the pol and core open reading frames. If expression of the core–pol fusion protein is deleterious to the host hepatocytes, one would expect a lower frequency of 3n+1 mutation. However, the replication behavior of HBV pDEL85-3n+1 appears to be similar to pDEL85-3n and pDEL85-3n+2. It is possible that the core–pol fusion protein is highly unstable in vivo, since we can detect wild-type core protein, but not the predicted core–pol fusion protein by immunoblot analysis (data not shown). It is also worth mentioning that Gunther et al. (2000) measured the production of polymerase in four HBV in-frame CID variants and found no consistent correlation between polymerase production and replication of CID variants. At present, it remains unclear what kind of selective pressures could account for the infrequent occurrence of 3n+1 deletion in this particular woodchuck No. 107. Additional mutations outside the core gene (e.g., see Ou, 1997) can in theory contribute to the relative abundance of these variants in different animals.

DI particles are not dominant negative mutants

It is perhaps useful to draw a distinction between the terminologies of DI particles and dominant negative mutants. The major distinction resides in the "enrichment" property which is lacking in dominant negative mutants. In other words, a DI particle is a conditional replicon. It can outcompete the helper viruses by preferential replication. By contrast, a dominant negative mutant can produce an inhibitory protein which can interfere with the normal functioning (e.g., replication) of the wild-type counterpart. However, such an inhibitory protein could not spare the dominant negative mutants themselves from being inhibited by their own protein. For example, an artificial core–surface fusion protein has been engineered and demonstrated to have a dominant negative effect on the replication of wild-type HBV (Scaglioni et al., 1994). This type of dominant negative mutants cannot replicate, enrich, and perpetuate themselves, even in the presence of helper viruses.

Can the internally deleted core protein of CID variants function like a repressor?

Similar to the dominant negative concept, one hypothesis to explain the mechanism of HBV CID interference is that the deleted core protein can function like a repressor which can inhibit wild-type HBV replication. According to this hypothesis, expression of such a putative repressor or inhibitory protein alone, but not the replication of CID variants per se, is important for the interference phenomenon. Our previous study is not in favor of this hypothesis since the internally deleted core protein was shown to be highly unstable in vivo and could not suppress wild-type HBV replication (Yuan et al., 1998a). The fact that DEL85-3n+1 and DEL85-3n+2 are as interfering as DEL85-3n (Fig. 1D) also suggests that the exact composition of the deleted core protein is probably not relevant to the interference phenomenon. Instead, presumably, it is the pregenomic RNA that is important for the DI phenomenon.

Defective interfering genome vs defective interfering particles

The conventional way to demonstrate defective interference is by in vitro infection and plaque assay (Huang and Baltimore, 1970; Holland et al., 1987; Dimmock, 1996). However, there is no plaque assay for HBV and a reproducible in vitro HBV infection system is still not readily available in this field. Our current approach of transfection, instead of the conventional approach of infection, has demonstrated a defective interfering genome of HBV CID variants. Based on our previous study, in the presence of a wild-type core protein, such a DI genome can be packaged and secreted in a manner indistinguishable from the wild-type HBV as shown by isopycnic gradient analysis (Yuan et al., 1998a). We predict here that such HBV DI-like particles should be as infectious as the wild-type HBV in vivo since they “borrow” the wild-type core protein from the helper virus, and their polymerase and envelope proteins are also of wild-type version. Indeed, one of such defective interfering
genomes (DEL85) has been found to be present, presumably in viral particles, in the sera from several patients in different countries (Yuan et al., 1998b).

Chronicity and pathogenicity

All three samples positive for WHV CID variants were from older chronic WHV carriers with HCC at necropsy. However, there are many other woodchucks of similar age and with HCC that are found to have no detectable CID variants in their sera. Lu et al. (2001) also reported the lack of association between HCC formation and the presence of CID variants. Similar to the association of HBV CID variants with chronic carriers, the WHV CID variants were also found in some of the woodchucks with persistent WHV infection. The low frequency of occurrence of WHV CID variants in WHV carriers appears to be consistent with a previous study that defective virus is not required for the establishment of persistent WHV infection (Miller et al., 1990). However, the distinction between defective and defective interfering viruses should be noted here. Replication-defective variants may not necessarily be interfering for wild-type virus. It is possible that multiple independent parameters (e.g., host or environmental factors) could contribute to the establishment of chronic infection. DI particles are known to be capable of modulating the course of virus-associated disease (Huang and Baltimore, 1970; Holland, 1987; Dimmock, 1996), rather than being a passive passenger or parasite. It is therefore interesting to investigate further if HBV CID variants can modulate the disease course in humans using the woodchuck model.

MATERIALS AND METHODS

WHV-infected woodchucks in the northeastern United States

Serum samples were collected from a total of 55 woodchucks chronically infected with WHV that were trapped in their native habitat between 1981 and 1987. Four woodchucks were trapped in Tompkins County, New York and two were from Fort Meade Army Base in Maryland. The remaining 49 samples from woodchucks were obtained from commercial sources and were trapped in Maryland or Delaware where WHV infection is hyperendemic and where certain areas have carrier rates as high as 80%. Serum samples were maintained at −70°C. Total DNA from serum samples was prepared as described previously (Hosono et al., 1995).

Amplification and cloning of WHV core gene via PCR

One aliquot of the extracted DNA sample was used as a template for the amplification of the core antigen region of WHV DNA. Two forward primers used for amplifying the core region were from nt 1979 to 1998 (5′ ACT TTT CAA GCC TCC AAG CT 3′ and 5′ ACT GTT CAA GCC TCC AAG CT 3′). These two primers differed from each other only in the fourth nucleotide from the 5′ end. The reverse primer was from nt 2611 to 2592 (5′ TTA GTT TTA TGT ACC CAT TG 3′). In subsequent screening, more forward (nt 1951–1971 5′ CCT TGT TTT TGC CTG TGT TCC 3′; nt 1906–1923 5′ ATA AAT GCA TGC GAC TTC 3′) and backward primers (nt 2647–2628 5′ TGA GCA GCT TGG TTA GAG TA 3′; nt 2634–2617 5′ TAG AGT AAA GAC CTG TAA 3′) were used. The amplification reaction was performed as described previously (Hosono et al., 1995) with the following modification. Depending on the volume of the PCR reaction (10 or 50 μl, respectively), different experimental conditions were used. Initial denaturation of the DNA sample at 94°C for 20 or 40 s was followed by 35 or 40 cycles of denaturation for 1 or 10 s at 94°C, annealing for 1 or 10 s at 60°C, and extension for 40 s at 70°C in a thermocycler (Idaho Tech Co.). The PCR-amplified DNA of about 600 bp in size was purified through 1.0% low-melting agarose gel. DNA was further purified via phenol/chloroform extraction and ethanol precipitation. Standard subcloning procedures were followed using the pGEM-T vector (Promega Co., Madison, WI). All the nucleotide numberings were used following the published WHV sequences by Cohen et al. (1988).

Plasmid constructs

(i) pCMW82-3n, pCMW82-(3n+1), and pCMW82-(3n+2). WHV CID mutants were generated by replacing a wild-type HindIII–BglII fragment of 337 bp in the core gene of plasmid pCMW82 (Seeger and Maragos, 1989) with the deleted version of HindIII–BglII fragments amplified by PCR from WHV DNA in woodchuck sera. Plasmid pCMW82-3n contains the in-frame deletion in the core region spanning from nt 2266 to 2400, whereas plasmids pCMW82-(3n+1) and pCMW82-(3n+2) have the out-of-frame deletions spanning from nt 2266 to 2363 and nt 2281 to 2417, respectively (Fig. 3A). These plasmids were confirmed by DNA sequencing.

(ii) pCMV-WHc. To construct the plasmid pCMV-WHc which expresses WHV core protein under the cytomegalovirus immediate-early promoter (CMV-IE), WHV core gene in the plasmid pCMW82 was PCR-amplified using an upstream 30-mer primer, 5′-GCGGAATTCCGCTTT-GGGGCGATGGAATGAT3′ with an EcoRI cleavage site (underlined), and a downstream 30-mer primer, 5′-GGCC-TCTAGATGTTTATTGATCC-ACTGTA3′ with an XbaI cleavage site (underlined). One nanogram of plasmid pCMW82 was used as a template DNA and 125 ng of each primer was added in a 10 μl PCR reaction consisting of a 94°C denaturing step for 40 s, followed by 35 cycles of amplification at 94°C for 5 s, 53°C for 5 s, and 72°C for 40 s. The amplified fragments were digested with EcoRI and XbaI enzymes and then subcloned into EcoRI and XbaI sites of the vector pCDNA3 (Invitrogen Co., USA).
(iii) pWT, pDEL85, pDEL85-(3n+1), and pDEL85-(3n+2). pWT was described as pSV2NeoHBV2X in Shih et al. (1989). pDEL85 is the same as pDEL85-(3n) which contains an in-frame core internal deletion (Yuan et al., 1998a). To construct HBV CID variants pDEL85-(3n+1) and pDEL85-(3n+2), two mutant primers were used in the Kunkel site-directed mutagenesis (Yuan et al., 1995): one 21-mer primer (5′ GAATTAGTAGCATGTGCCC-3′) created a 1-nt deletion and another 24-mer primer (5′ GAATTAGTAGCATGTGCCCTAT-3′) created a 2-nt deletion in pDEL85 (Fig. 1A). The mutations were confirmed by sequencing. Finally, these resulting monomers were dimerized in tandem in pSV2ANeo vector to construct the plasmids pDEL85-(3n+1) and pDEL85-(3n+2).

DNA transfection and preparation of intracellular core particle-associated viral DNA

DNA transfection into a human hepatoma cell line, Huh7, was conducted by calcium phosphate method (Shih et al., 1989) and intracellular core particle-associated viral DNA was isolated as described (Yuan et al., 1998a).

32P-labeled DNA probes used for Southern analysis

(i) WHV full-length probe. The full-length WHV DNA fragment of 3.3 kb was isolated from the plasmid WH81 (Kodama et al., 1985) digested with EcoRI, radiolabeled by [32P]dCTP using random priming kit (Boehringer Mannheim Co.).

(ii) WHV wild-type specific probe. The deleted region was PCR-amplified in the presence of [32P]dCTP using a 21-mer upstream primer, 5′ TCATGTCATTGATACCTGGGG and a downstream 21-mer primer, 5′ CTGATCATACCTACAACTT 3′. One nanogram of plasmid pCMW82 as a template, 125 ng of each primer, 1 μM of a mixture containing 200 mM each of dATP, dGTP, and dTTP, 1 μl 10× PCR buffer (Idea buffer containing 20 mM MgCl2), and 2.5 U Taq I polymerase were taken in an initial denaturation step at 94°C for 40 s, followed by 35 cycles of amplification at 94°C for 10 s, 53°C for 10 s, and 72°C for 40 s. The radiolabeled amplified DNA was used as a probe in Southern hybridization.

(iii) HBV full-length 3.1-kb probe, HBV wild-type specific probe, and HBV CID-specific probe. These probes were prepared as described previously (Yuan et al., 1998b).

Sequence analysis of recombinant WHcAg clones

Sequencing reactions for the cloned PCR products in both sense and antisense directions were conducted using the Sequenase kit (U.S. Biochemical Corp., Cleveland, OH). The PCR primers described above were used in the DNA sequencing analysis. At least five independently isolated recombinant clones per sample were sequenced. The WHcAg DNA sequences were translated by a computer program into their corresponding amino acid sequences and then aligned with consensus WHV sequences (Galibert et al., 1982; Kodama et al., 1985; Cohen et al., 1988; Girones et al., 1989).

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REFERENCES


