Enhanced Replication Contributes to Enrichment of Hepatitis B Virus with a Deletion in the Core Gene

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Accumulation in immunosuppressed patients of hepatitis B virus (HBV) with a deletion in the C gene is associated with severe liver disease. The aim of this study was to determine the phenotype of such genomes *in vitro.* Four C gene fragments with different types of deletions were inserted in the context of a wild-type genome and tested by transfection into HuH7 cells. The deletions did not influence mRNA and surface protein levels. Truncated C gene translation products were expressed only from variants with in-frame deletions, whereas full-length polymerase was expressed from all variants at a similar or higher level than in wild-type virus. None of the variants was competent for autonomous replication; however, they produced 2- to 4.5-fold more progeny DNA than wild-type HBV when sufficiently complemented with wild-type core protein. Similarly, when variant and wild-type DNA were cotransfected in different ratios, the variants produced 2- to 5-fold more progeny DNA relative to the wild-type; this enrichment required the expression of the viral polymerase *in cis.* The mechanism of enrichment depended on the percentage of variant in the transfected DNA mixture. When the transfected DNA contained a small percentage of variant, enhanced replication of the variant accompanied by no or little suppression of wild-type replication was seen. Accordingly, overall production of progeny virus was slightly increased. At a high percentage of variant DNA, replication of both variant and wild-type decreased, probably due to a shortage of wild-type core protein. In conclusion, emergence of C gene deletion variants *in vivo* may be due to enhanced replication mediated at the level of encapsidation or reverse transcription. If the variants constitute a small part of the ccc DNA, they can be fully *trans*-complemented by wild-type virus which may increase the overall virus production.   © 2000 Academic Press

INTRODUCTION

Hepatitis B virus (HBV) variants are found in patients with acute and chronic infection. Many variants are predominantly seen in particular stages of chronic infection (Günther *et al.,* 1999). Those with large deletions in the middle of the nucleocapsid gene (C gene deletion variants) seem to emerge in the early phase of chronic infection, characterized by the presence of hepatitis B e antigen (HBeAg) in serum and normal alanine aminotransferase levels (Okamoto *et al.,* 1987; Tsubota *et al.,* 1998). But they are likely to persist only temporarily. Their presence in the circulation was found to predict seroconversion from HBeAg to the corresponding antibody (anti-HBe) in the near future and, following this event, the variants frequently disappear from circulation (Wakita *et al.,* 1991; Marinos *et al.,* 1996). According to these longitudinal data, C gene deletion variants were often demonstrated in HBeAg-positive patients (Ackrill *et al.,* 1993;

Fiordalisi *et al.,* 1994; Uchida *et al.,* 1994; Akarca and Lok, 1995; Okumura *et al.,* 1996; Zoulim *et al.,* 1996) but rarely in those who were negative for HBeAg (Takayanagi *et al.,* 1993; Horikita *et al.,* 1994; Nakayama *et al.,* 1995). The variants were found in few patients with hepatocellular carcinoma (Hosono *et al.,* 1995). Emergence and accumulation of C gene deletion variants was also observed in HBeAg-positive renal transplant recipients after long-term immunosuppression (Günther *et al.,* 1996a). In contrast to the natural course of infection, persistence of the variants in these transplant patients was associated with the development of end-stage liver disease and death of patients from liver disease (Günther *et al.,* 1996a).

All C gene deletion variants tested *in vitro* so far were found to be defective for autonomous replication (Okamoto *et al.,* 1993; Yuan *et al.,* 1998b). In these studies genome replication was rescued when nondeleted (referred to as wild-type) core protein was supplied *in trans* (Yuan *et al.,* 1998b). Quantitative analysis of the replication level and its dependence on the amount of supplied wild-type core protein was not performed. Cotransfection of wild-type HBV genomes with viruses containing the C gene deletion resulted in a strong suppression of wild-type HBV replication and an enrichment of progeny virus with deletions in the C gene (Yuan *et al.,* 1998a). This phenotype led the authors to conclude that HBV with

deletions in the C gene behaves like defective interfering particles similar to that observed in other virus-cell systems. However, it is not clear from this study whether this phenotype was due to the deletions in the C gene alone as all tested variants also contained mutations in the core promoter previously shown to enhance replication (Buckwold *et al.,* 1996). Furthermore, unlike what was expected from this study, accumulation of C gene deletion variants in patients appears not to be associated with decreased viremia (Wakita *et al.,* 1991; Günther *et al.,* 1996a; Tsubota *et al.,* 1998). Therefore, we hypothesized that the phenotype of these variants might be more complex than described and analyzed a representative set of C gene deletion variants without promoter mutations under various conditions in cell culture. It is demonstrated here that the C gene deletion variants can replicate at higher levels compared to HBV without a deletion in the C gene, when complemented *in trans* with the appropriate level of wild-type core protein or wild-type virus. We also show that the C gene deletion variants express similar or higher levels of polymerase protein as wild-type virus and are enriched when coreplicating with wild-type virus. Enrichment of the deletion variants appears to depend on both enhanced replication and suppression of the wild-type virus depending on the percentage of cotransfected wild-type virus, indicating that takeover of C gene deletion mutants *in vivo* is more complex than previously speculated.

RESULTS

Cloning and characterization of C genes with deletions and construction of hybrid genomes for phenotypic analysis

HBV DNA was isolated from the serum of four previously described patients (Günther *et al.,* 1996a; Marinos *et al.,* 1996) and the C gene was amplified by PCR. C gene deletion variants in all four patients represented a major virus population as indicated by the substantial fraction of amplified fragments which were shorter than wild-type length (Fig. 1A). Cloning and sequencing of several amplified C genes revealed that patients I, D, G, and FT were infected by HBV with a deletion of 90, 24, 120, and 144 nucleotides in the C gene, respectively. Clones with the 144-nucleotide deletion had additionally a TGG → TAG change in codon 28 of the pre-C region preventing HBeAg expression and an insertion of a singe T residue after position 1950. For testing the functional consequences of C gene deletions, a mutant C gene fragment of each patient was inserted into the context of a wild-type genome by fragment exchange, which resulted in hybrid genomes ΔC-90, ΔC-24, ΔC-120, and ΔC-144 (Fig. 1B). Constructs ΔC-90 and ΔC-24 with inframe deletions may express core protein and HBeAg with an internal deletion, while ΔC-120 and ΔC-144 can only express short N terminal core peptides, because of
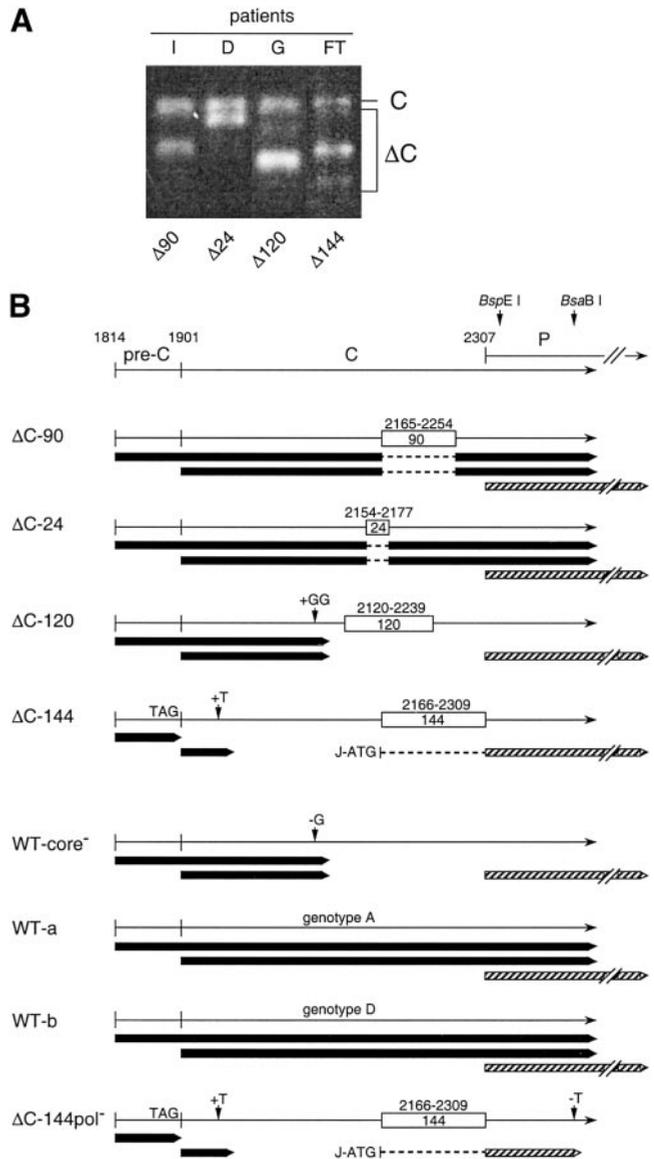

FIG. 1. (A) The C gene of HBV from serum of four patients was amplified and separated in 2% agarose gel (C, wild-type C gene; ΔC, C gene with deletion). The number of deleted nucleotides as determined by sequencing is shown below the gel. (B) Schematic presentation of the C gene and the overlapping P gene of deletion variants and control virus used in the study. The restriction sites used for exchange of the mutated fragments with the corresponding C gene fragment of pHBV-SapI are indicated at the top. Deletions are shown as open boxes with the number of deleted nucleotides within the boxes and the position of the deleted sequence above the boxes. Single nucleotide deletions (−) and insertions (+) are indicated by arrows. Predicted translation products starting at the pre-C-ATG (position 1814), the C-ATG (position 1901), the P-ATG (position 2307), and the J-ATG (position 2163, for ΔC-144 and ΔC-144pol⁻) are indicated by large arrows below each construct.

upstream insertions. The deletions in ΔC-90, ΔC-24, and ΔC-120 do not affect the polymerase gene, whereas the deletion in ΔC-144 removes the authentic polymerase start codon and fuses the polymerase gene with an upstream located ATG, designated previously J-ATG (position 2163–2165) (Fouillot *et al.,* 1993).
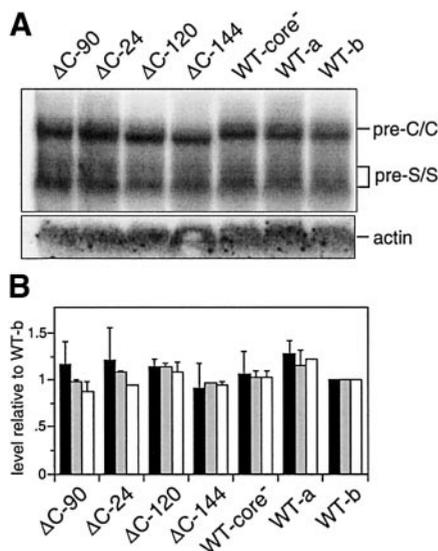
FIG. 2. (A) RNA blot analysis of the major HBV transcripts and actin RNA as a control. (B) Quantitative evaluation of the pre-C/C RNA levels (filled bar), the ratio of the pre-C/C to pre-S/S RNA levels (shaded bar), and the actin RNA levels (open bar) as determined in two transfection experiments (mean and standard deviation).

Four control genomes were used for phenotypic analysis (Fig. 1B). The genotype D wild-type genome (WT-b) used for construction of all hybrid genomes was chosen as the main reference in all experiments. The WT-a hybrid genome was constructed to test whether genotype-A-specific mutations, as present in the C gene of ΔC-90, ΔC-24, and ΔC-120, influence the phenotype. Hybrid genome WT-core⁻ served as a control to test whether a defect in core protein production is important for the phenotype of the deletion variants. It encodes N-terminal core and HBe peptides identical to those encoded by ΔC-120, but does not contain a large deletion in the C gene. Control genome ΔC-144pol⁻ is identical to ΔC-144, but cannot express polymerase due to an artificial frameshift mutation in the amino terminal end of the P gene. It was constructed in order to study whether the expression of polymerase *in cis* is essential for the phenotype of the deletion mutants.

All genomes were cloned as monomers. For functional testing, the genomes were released from the plasmid by *Sap*I digestion and transfected into HuH7 cells (Günther *et al.,* 1995). This method has been successfully used for phenotypic characterization of various types of HBV variants (Günther *et al.,* 1996b, 1998; Sterneck *et al.,* 1998a,b).

Viral mRNA levels

The HBV RNA levels were analyzed by Northern blot of total cellular RNA. Hybridization of actin mRNA served as a control for the amount of RNA loaded onto the blot. A representative blot is shown in Fig. 2A, and the quantitative analysis of two independent experiments is shown

in Fig. 2B. All major HBV RNA species were produced in similar amounts from the deletion variants and the control genomes. As expected, the 3.5-kb pre-C/C gene transcripts of the deletion mutants were slightly shorter due to the deletion. There was also no difference in the ratio of pre-C/C gene to pre-S/S gene transcripts. These data suggest that the deletions in the C gene do not interfere with transcription or the half-life of the major viral RNAs.

Protein expression

Corresponding to the unchanged level of pre-S/S mRNA, all genomes produced similar amounts of pre-S1 and pre-S2 protein, as detected by immunoblot analysis of total cellular lysate (Fig. 3A), and secreted similar amounts of hepatitis B s antigen (HBsAg) into the cell culture supernatant, as measured by enzyme immunoassay (Fig. 3B). Since the deletions in the C gene did not affect the synthesis of surface RNA and protein, the surface protein level was used as an internal standard for quantification of other virus products in subsequent experiments.

Proteins expressed from the C gene were detected for both variants—ΔC-90 and ΔC-24—with in-frame deletion. A high level of HBeAg was measured by enzyme immunoassay in the cell culture supernatant of ΔC-24-transfected cells, whereas a level close to background was found for ΔC-90 (Fig. 4A). As expected, HBeAg was not detected for ΔC-120, ΔC-144, and WT-core⁻, which
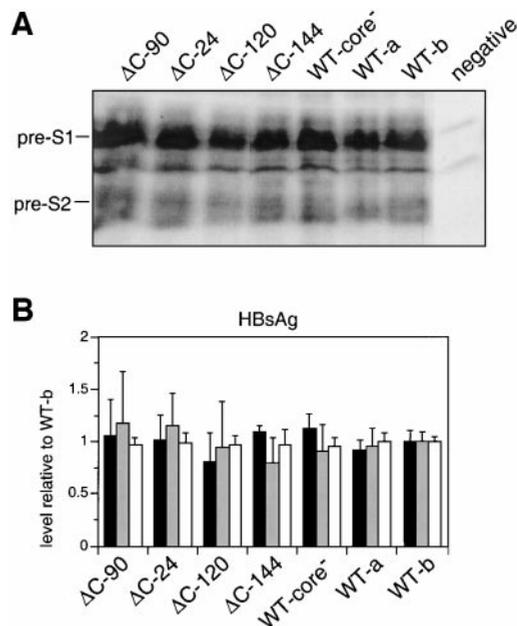
FIG. 3. (A) Immunoblot analysis of pre-S1/2 proteins. (B) HBsAg levels in the supernatant as detected by commercial enzyme immunoassay (mean and standard deviation; black bars, variants transfected alone, $n = 5$; shaded bars, variants cotransfected with pCMV core, $n = 9$; open bars, variants cotransfected with WT-b, $n = 6$).
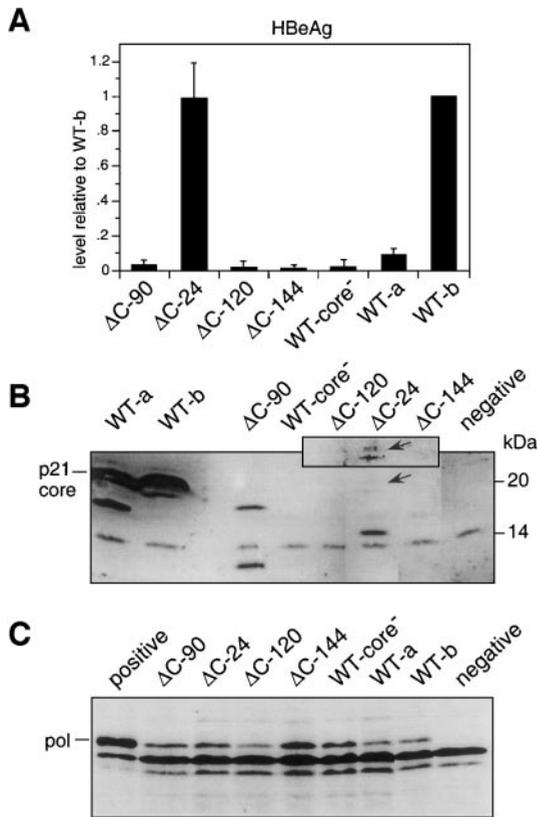
FIG. 4. (A) HBeAg levels in the supernatant as detected by commercial enzyme immunoassay (mean and standard deviation, n = 10). Differences between the HBe amino acid sequence of WT-a and WT-b probably led to reduced secretion and/or antigenicity of WT-a. (B) Immunoblot analysis of pre-C/C gene translation products. The position of core protein of wild-type length is indicated. The arrows indicate the position of a double band which is faint in the large image but clearly visible in the inlet with enhanced contrast. (C) Immunoblot analysis of polymerase. The position of polymerase is indicated. Positive control, cells transfected with a polymerase expression plasmid.

cannot express HBeAg. Consistent with the immunoassay data, ΔC-90 and ΔC-24 expressed shortened C gene translation products as detected by immunoblotting of whole cell lysate using an antibody that was raised against denatured core protein and recognizes several linear epitopes scattered over the core protein sequence (Bichko et al., 1993) (Fig. 4B). The upper and lower bands are likely to correspond to core and HBe protein, respectively, with internal deletion. However, compared to the core protein of both wild-type constructs, the putative core protein of ΔC-90 and ΔC-24 was clearly less well detectable. Taking into consideration that most of the epitopes recognized by the antibody are present on the shortened core protein species, the data suggest that the internal deletions strongly reduce the steady-state level of the protein.

As tested by immunoblot analysis of whole cell lysate, all deletion mutants expressed a full-length polymerase protein (Fig. 4C) even if the authentic start codon was replaced as in ΔC-144. No obvious changes in the poly-

merase steady-state level were observed for variants with deletions upstream of P-ATG, whereas the polymerase signal of ΔC-144 was clearly stronger compared with that of the wild-type genomes. Quantitative evaluation of two separate blots revealed consistently a threefold increase that was also evident when the pre-S1/2 protein level measured on the same blots was used for standardization of the polymerase values of ΔC-144.

Replication capacity of C gene deletion variants upon complementation with wild-type core protein

HBV genomes were transfected into HuH7 cells and core particle-associated progeny HBV DNA was isolated from cytoplasm and cell culture supernatant and subjected to Southern blot analysis. All deletion variants as well as WT-core⁻ were not competent for autonomous replication (Fig. 5A, left). Their replication was rescued upon cotransfection of a plasmid expressing wild-type core protein under CMV promoter control (Fig. 5A, middle and right). The replication level of the variants depended on the amount of core protein supplied in trans. The addition of 0.4 μg of core protein expression plasmid per 1 μg of HBV DNA resulted in progeny DNA levels similar to or slightly above that of WT-b (Fig. 5B). Increasing the amount of expression plasmid to 1.33 μg per 1 μg of transfected HBV DNA increased the replication level of the deletion variants about 2-fold compared to WT-b. Quantitative evaluation of the signals of four transfection experiments revealed that the deletion variants produced 2- to 4.5-fold more intracellular replicative intermediates and 1.8- to 2.8-fold more extracellular HBV DNA than WT-b and WT-core⁻ (Fig. 5B). When compared with WT-a, the factors were even higher. Note that WT-a and WT-b have a higher intracellular level of wild-type core protein available than the variants, as both were also cotransfected with the same amount of expression plasmid. Taken together, the C gene deletion variants were not competent for autonomous replication, but replicated more than wild-type HBV when complemented with sufficient amounts of wild-type core protein. The enhanced replication of the variants is specifically due to the deletion in the core gene rather than to the defect in core protein expression, as is evident from the normal replication level of WT-core⁻ complemented with wild-type core protein.

Enrichment of deletion variants after cotransfection of variant and wild-type DNA

Because the deletion variants replicated more efficiently than wild-type HBV upon complementation with core protein, we tested whether they can overgrow wild-type virus. To this end, both virus DNAs were cotransfected into cells and the ratio of variant to wild-type progeny DNA in cytoplasm and supernatant was compared with the ratio of variant to wild-type template DNA
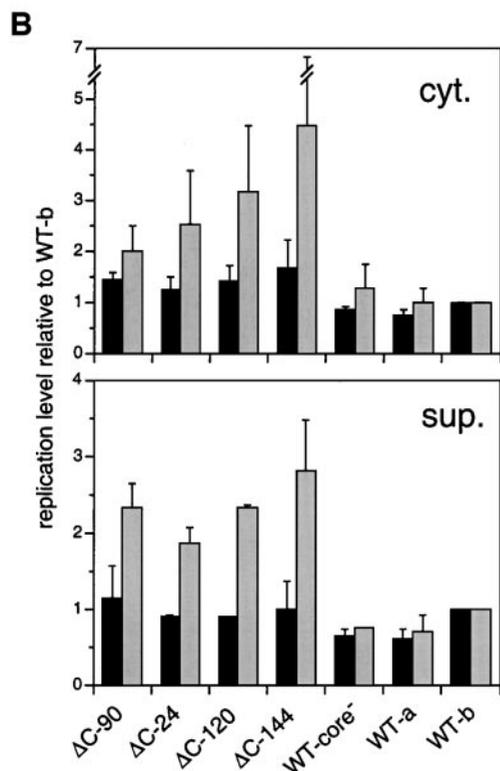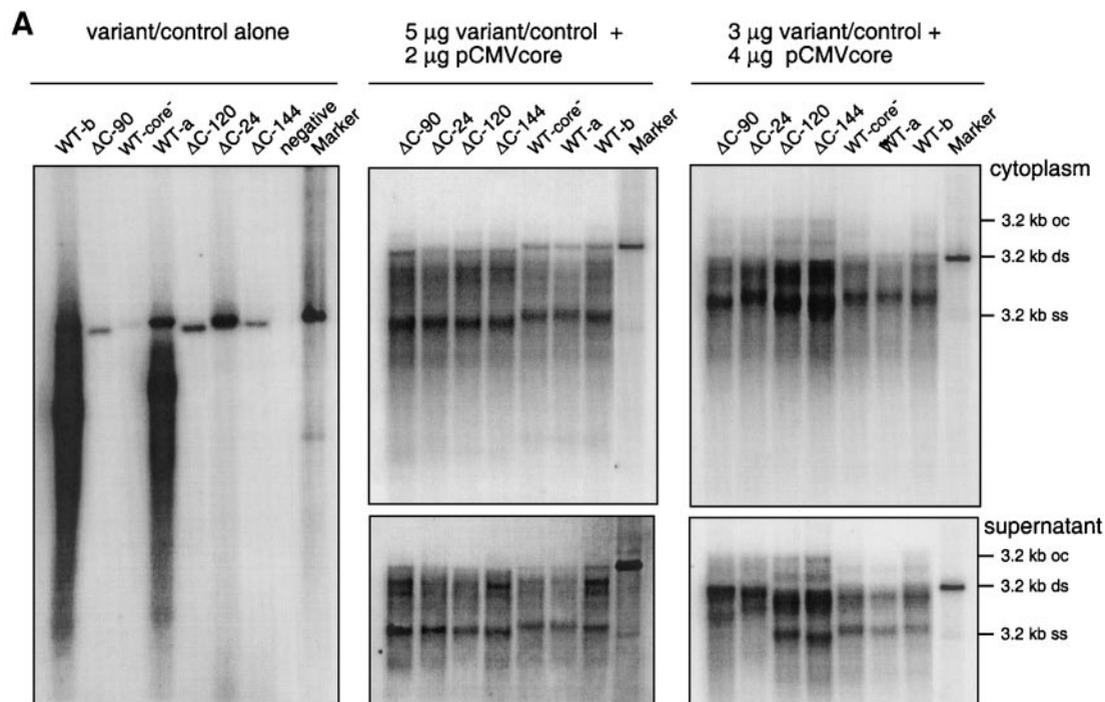
FIG. 5. (A) Representative Southern blot analysis of particle-associated replicative HBV DNA produced by variants and controls with or without cotransfected pCMV core. The marker contains a 3.2-kb double-stranded (ds) and a 3.2-kb single-stranded (ss) fragment. The positions of these fragments as well as of open circular HBV DNA (oc) are indicated at the right. On the left, signals at the double-strand position represent input HBV DNA. Note that the ss HBV DNA runs in some lanes at different positions due to variation of salt concentrations in individual samples. (B) Level of replicative HBV DNA standardized with the corresponding HBsAg values (mean and standard deviation; black bar, 5 μg variant cotransfected with 2 μg pCMV core, $n = 2$; shaded bar, 3 μg variant cotransfected with 4 μg pCMV core, $n = 4$). Top, replicative DNA level in cytoplasm; bottom, replicative DNA level in supernatant.
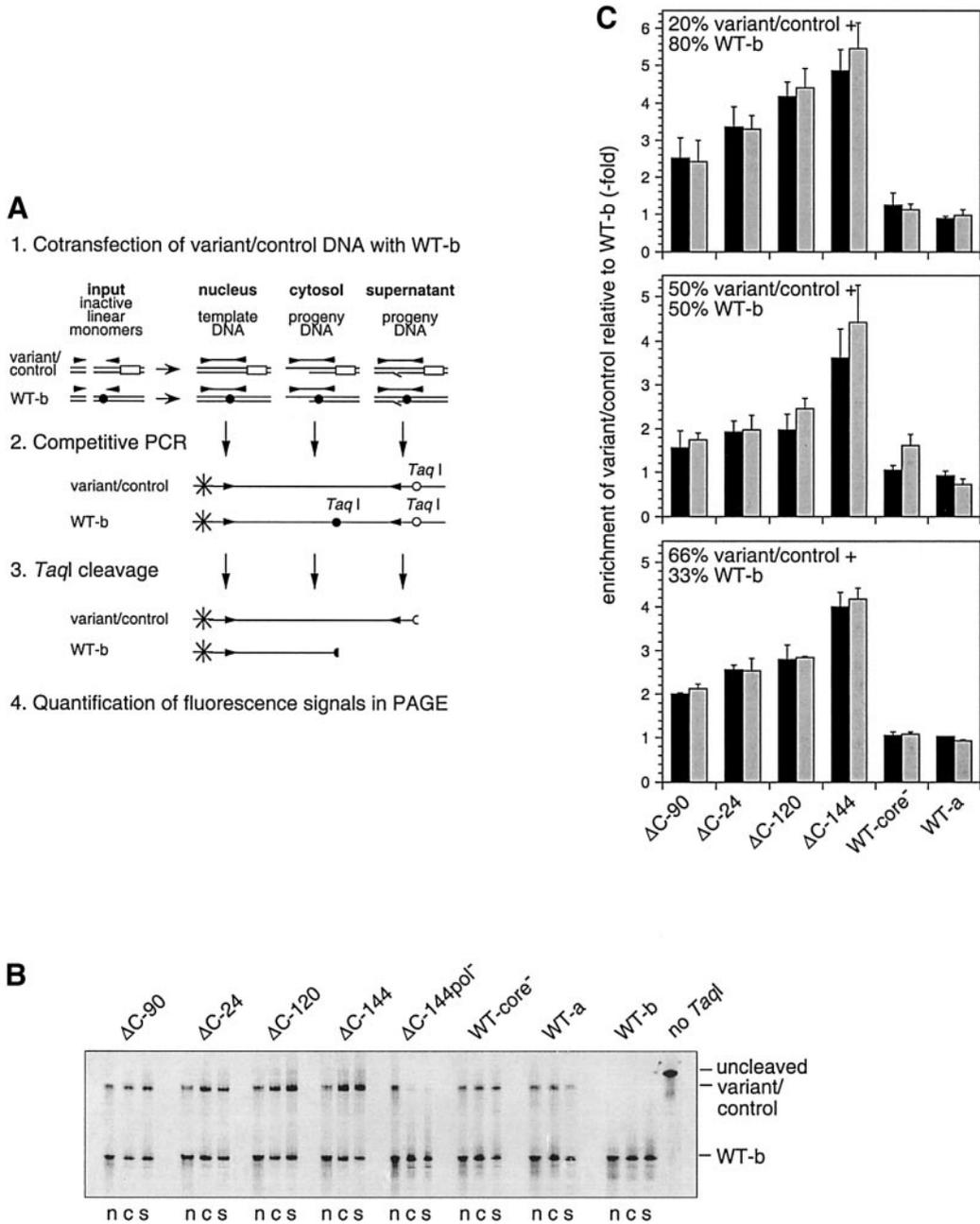
FIG. 6. (A) Schematic presentation of the assay for measurement of enrichment of C gene deletion variants. Primers are indicated by small arrows, the fluorescence dye by an asterisk, the internal *Taq*I marker mutation by a black dot, the control *Taq*I site by an open dot, and the C gene deletion by an open box. (B) Representative polyacrylamide gel analysis of the dye-labeled PCR products amplified with HBV DNA from nucleus (n), cytoplasm (c), and supernatant (s) following 50%/50% cotransfection of variant and WT-b. variant/control, position of the cleaved PCR products of variants and controls; WT-b, position of the cleaved PCR products of WT-b. (C) Enrichment factor in cytoplasm (black bars) and supernatant (shaded bars) as determined with the assay for different percentages of variant or control in the transfected DNA mixture. Mean and standard deviation of at least two determinations.

in the nucleus. A higher variant/wild-type ratio in the progeny DNA than in the nuclear DNA indicates more efficient replication of the variant relative to wild-type, which is designated here as "enrichment." The assay to measure these ratios is based on competitive coamplification of variant and wild-type DNA followed by quantitative restriction fragment length polymorphism (RFLP) analysis (Fig. 6A). In detail, after cotransfection of a

deletion variant or control virus with WT-b, HBV DNA was purified from nucleus, cytoplasm, and supernatant and amplified by PCR. A problem with PCR analysis of HBV DNA produced following transfection is contamination of the DNA preparation with input DNA (for an example of such contamination see Fig. 5A, left). In this respect, the use of linear monomeric HBV genomes for transfection is of great advantage. An appropriate primer design can

prevent amplification of input DNA unless it is converted into replication-competent templates in the nucleus, which is not possible if plasmid-integrated overlength HBV genomes are used for transfection. Therefore, the primers chosen for PCR encompass a region which is not contiguous on the transfected linear DNA, namely the nick region of the HBV genome. Furthermore, the deletions in the C gene are not located within the amplicons to prevent preferential amplification of the variant DNA. The amplicons of the deletion variants and controls were distinguished from those of WT-b by RFLP analysis using TaqI enzyme. A corresponding site is unique in WT-b amplicons (position 1909) but absent from amplicons of variants and controls. As an internal control for cleavage, a second TaqI site was engineered at the 5′ end of one PCR primer followed by a heterologous tail of 15 nucleotides. Failure of the cleavage assay would result in incomplete removal of this short sequence from the PCR products. Following TaqI cleavage, the PCR products were subjected to denaturing polyacrylamide gel electrophoresis to separate fragments which remained uncleaved, those of the variants which were cleaved only at the control site, and those of WT-b which were cleaved both at the control and at the internal sites (positions uncleaved, variant/control, and WT-b, respectively, in Fig. 6B). The fragments were detected and quantified via a fluorescence dye coupled to one of the PCR primers. In all assays that were performed, no fragments at the uncleaved position remained (as an example, see Fig. 6B) indicating that all available TaqI sites were cleaved. The variant/WT-b signal ratio in the nucleus, cytoplasm, and supernatant was determined and the enrichment factor was calculated as described under Materials and Methods.

Three sets of cotransfection experiments were performed to test the enrichment of the variants. Mixtures of 20% variant or control with 80% WT-b DNA, mixtures of 50% variant or control with 50% WT-b DNA, and mixtures of 66% variant or control with 33% WT-b DNA were transfected. In all three sets of experiments, the variants became enriched 1.5- to 5.1-fold in the cytoplasm and 1.8- to 5.3-fold in the supernatant (Fig. 6C). Consistent with the core protein complementation experiments, no enrichment was seen for WT-a and WT-core⁻. The enrichment factors for the deletion variants correlated fairly well with their replication level observed upon supply of wild-type core protein by pCMV core in the order ΔC-90 < ΔC-24 < ΔC-120 < ΔC-144, suggesting that both effects are linked. In contrast to ΔC-144, the control variant ΔC-144pol⁻, which is identical to variant ΔC-144 but does not express polymerase, was completely overgrown by WT-b (Fig. 6B, lane ΔC-144pol⁻). The extremely weak PCR signal for ΔC-144pol⁻ DNA in cytoplasm and supernatant confirms the highly *cis*-preferential packaging of pregenomic RNA by the HBV polymerase (Bartenschlager *et al.,* 1990). In addition, it shows that there is no

contamination of the cytoplasmic preparation by nuclear components and no contribution of linear input DNA to the PCR signal. In conclusion, consistent with the core protein complementation analysis, C gene deletion variants produce more progeny DNA than wild-type HBV when both viruses coreplicate. This effect is independent of the percentage of variant in the transfected DNA mixture. A defect in core protein synthesis is not important for enrichment as revealed by analysis of WT-core⁻, whereas expression of polymerase *in cis* is essential.

Overall and proportionate progeny DNA synthesis after cotransfection of variant and wild-type DNA

The enhanced replication of the variants may lead to an increased overall production of progeny virus after cotransfection of variants and WT-b, provided that WT-b produces sufficient core protein and there is no major inhibitory effect of the variants on wild-type replication. In order to test this hypothesis, progeny virus DNA in cytoplasm and supernatant that was produced following cotransfection of variant with WT-b was subjected to Southern blot analysis (Fig. 7A). The signals were quantified and the progeny DNA level—relative to that obtained with WT-b alone—was plotted versus the percentage of variant DNA in the cotransfected mixture. (Fig. 7B, top; data of the transfection of 100% variant or control shown in Fig. 5A, left, were also included). The resulting curves revealed a small increase in progeny DNA synthesis when the transfected DNA mixture contained 20 and/or 50% deletion variant, while no clear effects were observed with the control genomes WT-a and WT-core⁻. Although this increase is close to the difference that can reliably be detected in transfection assays, several facts argue for a biological effect. The increased virus production was observed with all four variants, was demonstrated in cytoplasm and supernatant, and was reproduced in several experiments. In addition, the strongest effect was observed with ΔC-144 which also showed the highest level of replication upon complementation with core protein. Altogether, these data indicate that a nuclear HBV DNA pool containing 20 to 50% C gene deletion variant produces slightly more progeny virus than a corresponding amount of DNA consisting only of wild-type HBV DNA.

The above experiments raised the question of whether the enrichment of C gene deletion variants and the increase of overall DNA synthesis result only from enhanced replication of the variants, or whether changes of the WT-b replication level play an additional role. To address this question, the proportionate progeny DNA level of variant and WT-b was calculated using the experimentally determined enrichment factor and the corresponding level of overall DNA synthesis. The values were plotted versus the percentage of variant DNA in the transfected DNA mixture. The resulting curves revealed

that all deletion variants produced a high level of progeny DNA (up to 1.3) even when their proportion in the transfection mixture was only 20 or 50% (Fig. 7B, bottom). Note that WT-b produces a progeny DNA level of 1.0 when it accounts for 100% of the transfected DNA. On the other hand, the synthesis of progeny WT-b DNA correlated roughly with the proportion of WT-b in the transfected DNA in these experiments (Fig. 7B, bottom). This indicates enhanced replication of the variants and largely unchanged replication of wild-type when the transfected DNA mixture contained 20 or 50% variant. To express this quantitatively, the progeny DNA level was set into a relation with the corresponding amount of transfected DNA. This revealed that the variants produced 1.5- to 5-fold more progeny DNA relative to the amount of transfected DNA, whereas the synthesis of progeny WT-b DNA corresponded to or was slightly reduced relative to the amount of transfected WT-b DNA (Fig. 7C, left and middle). The situation was different when 66% variant and 33% WT-b DNA were transfected (Fig. 7C, right). Here, the variants produced only marginally more progeny DNA, whereas the synthesis of progeny WT-b DNA was clearly reduced relative to the amount of transfected DNA. No considerable effects were seen with control constructs WT-core⁻ and WT-a. Altogether, these data indicate that at a low percentage of deletion variant in the nucleus, there is no or little interference between variant and wild-type; both replicate similarly as seen after complementation with sufficient amounts of core protein by pCMVcore. Accordingly, the slightly increased overall synthesis of progeny DNA and the enrichment of the variants are mainly the result of the variant's enhanced replication. When the percentage of variant DNA in the transfection mixture is high, the replication of both viruses is submaximal. Under these conditions, suppression of WT-b replication contributes to enrichment of the variants.

DISCUSSION

In this study the phenotype of HBV with deletions in the C gene and its interaction with wild-type HBV are described. A representative set of mutant C genes, derived from major virus populations of four patients, was analyzed in the context of a wild-type genome by transfection into HuH7 cells. The deletions in the C gene significantly altered neither virus RNA synthesis nor the expression of surface proteins. Shortened forms of C gene translation products were expressed by variants with in-frame deletions, whereas full-length HBV polymerase was expressed by all variants even if the authentic P gene start codon was removed. Although none of the variants was competent for autonomous replication, they showed enhanced replication when supplied with sufficient amounts of wild-type core protein in trans. Similarly, upon cotransfection with wild-type HBV the

variants became enriched independent of the percentage of cotransfected variant. The enrichment of progeny DNA of the variants is a complex phenomenon. At a low percentage of cotransfected variant it resulted mainly from enhanced replication of the variant, whereas at a high percentage suppression of wild-type replication contributed to enrichment. When the transfected DNA contained 20 or 50% variant, its enhanced replication also led to a small increase of the total progeny virus production.

Previous attempts to detect C gene translation products of variants with in-frame deletions in the C gene were not successful (Yuan et al., 1998b). By using an antiserum recognizing several epitopes on denatured core protein (Bichko et al., 1993), shortened species of C gene translation products could be demonstrated in our study. In agreement with a previous study, which suggested that core protein with internal deletions is unstable (Yuan et al., 1998b), the putative core protein, especially that of ΔC-24, was hardly detectable. However, unlike that reported for other C gene deletion variants (Yuan et al., 1998b), the level of HBeAg secreted by ΔC-24 was not reduced, indicating that the stability of HBeAg was not affected and that C gene deletion variants are not generally negative for HBeAg. Altogether, these results argue for the existence of protein sequences removed in ΔC-24 which are important for stability of cytoplasmic core protein, but not necessarily for stability of HBeAg, which is secreted via the endoplasmic reticulum. These sequences may either increase protein stability directly or facilitate assembly of core protein into stable particles. Instability of core protein and/or inability to form core particles are also likely reasons why HBV with in-frame deletions in the C gene—even if only eight amino acids are removed as in ΔC-24—is not competent for autonomous replication.

Variant ΔC-144 is an interesting example as in this variant the authentic polymerase start codon was removed and the so-called J-ATG was fused with the P gene. The expression of a full-length polymerase indicates that the J-ATG is actually used for translation. In addition, ΔC-144 seems to express more polymerase than wild-type HBV. Since the level of pre-C/C mRNA was not increased, it is probably the use of the J-ATG that leads to enhanced translation efficiency. This speculation is supported by two major arguments: first, compared with the authentic P-ATG, the J-ATG is in a context that predicts more efficient translation initiation by eukaryotic ribosomes (Kozak, 1986). Second, the translation of the polymerase, which is thought to occur via ribosomal scanning on the pregenomic RNA, is hampered in wild-type HBV by the upstream J-ATG (Fouillot et al., 1993). This translational downregulation cannot be functional in ΔC-144 as the J-ATG is used for polymerase expression.

An interesting question that arises from this study is
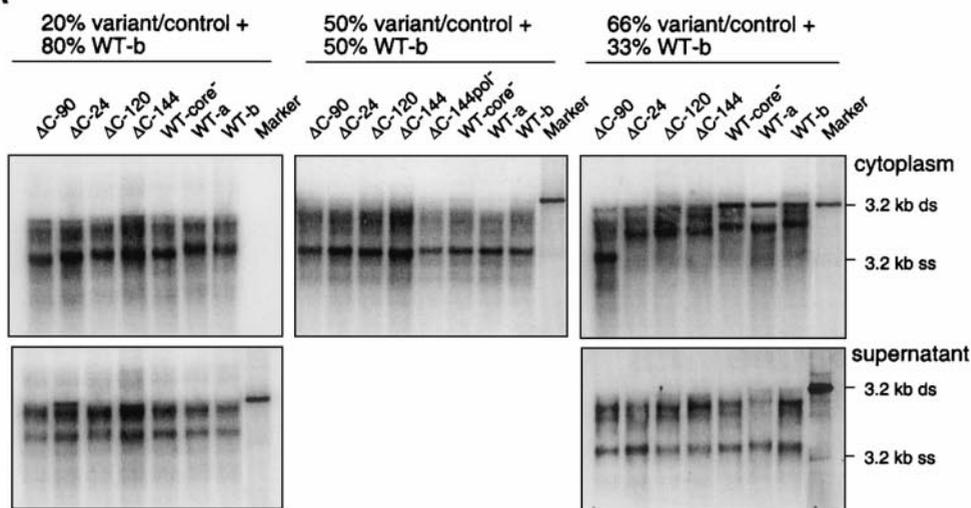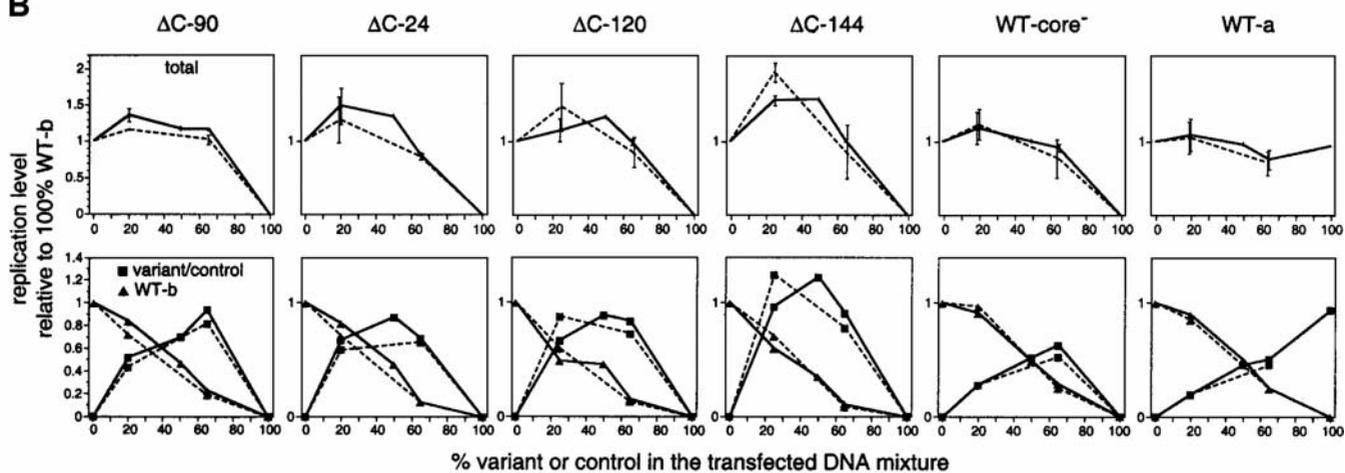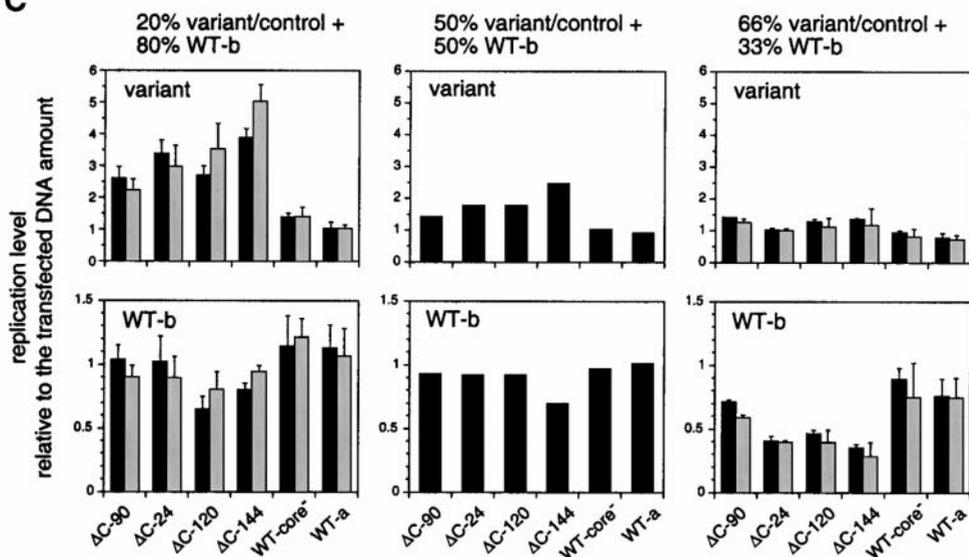
how deletions in the C gene eventually mediate enhanced replication and enrichment. The RNA data indicate that these effects are not due to enhanced transcription or stability of the pregenomic RNA. Therefore, the phenotype of the variants is likely to result from enhanced pregenome encapsidation or reverse transcription. It is conceivable that the affinity of the deleted pregenomic RNA to polymerase or core protein is increased. Alternatively, or in addition, the encapsidation may be increased because polymerase is more efficiently translated from the deleted pregenomic RNA, or the deletion in the encapsidated RNA facilitates the conversion into minus-strand DNA. Since RNA encapsidation and, thus, reverse transcription by the polymerase is highly *cis*-preferential (Bartenschlager *et al.,* 1990), these conclusions are also consistent with the importance of polymerase expression *in cis* for the phenotype. Increased translation of polymerase due to the use of the J-ATG is likely to contribute to the phenotype in variant ΔC-144. Provided that the removal of specific sequences mediates the phenotype of ΔC-24, ΔC-90, and ΔC-120, these sequences are probably located within positions 2165–2177, the common overlap of the three deletions.

The characteristic replication curves observed when C gene deletion variants and wild-type were cotransfected can be explained straightforwardly if one assumes an excess production of core protein by wild-type HBV. The question of how much core protein is produced by wild-type virus in relation to pregenomic RNA and polymerase has not yet been directly addressed. However, immunohistological and electronmicroscopical data of infected hepatocytes argue for an excess production of core protein. In some chronic HBV carriers as well as in HBV-transgenic mice that replicate the virus, the nucleus contains large amounts of core protein devoid of viral nucleic acids (Farza *et al.,* 1988; Phillips *et al.,* 1992; Guidotti *et al.,* 1995). Since in transgenic mice virus particles cannot enter the cell, this core protein is apparently produced in excess within the cell and is not required for synthesis of new virus. Therefore, it is likely that wild-type HBV also produces in HuH7 cells an excess of core protein which is sufficient for maximal replication of C gene deletion variant and wild-type virus, at least when the percentage of variant in the nuclear DNA pool is low, a condition which may be referred to as "noncompetitive" coreplication. Under this condition the variant produces more progeny DNA, as observed after supply of sufficient core protein by expression plasmid. As there is no or little concomitant suppression of wild-type replication, enrichment of the variants and the small increase in total virus production result mainly from the enhanced replication of the variants. This explains why the factors for enrichment and enhanced replication are very similar for each variant. When the percentage of deletion variant in the nuclear DNA pool increases above a certain threshold, the produced wild-type core protein is no longer sufficient for maximal replication of both viruses. Under these conditions both viruses compete for the limited amount of core protein which may be referred to as "competitive" coreplication. The overall virus production now decreases in parallel with the decreasing percentage of wild-type virus and, thus, amount of intracellular core protein. Although the shortage in core protein inhibits the progeny DNA production of both viruses compared with the noncompetitive phase (Fig. 7C), the variant still becomes enriched, probably because its pregenomic RNA is more efficiently encapsidated or reverse transcribed. In conclusion, the variants can replicate more efficiently than wild-type virus without significantly inhibiting its replication. However, if they constitute a large proportion of the virus population, wild-type virus replication appears to be indirectly suppressed due to core protein shortage.

The *in vitro* data have implications for understanding the selection and counterselection processes of C gene deletion variants *in vivo*. Because enrichment was independent of the percentage of variants in the nuclear DNA pool, one should expect that C gene deletion variants, once they appear in the virus population of a patient, almost completely replace wild-type HBV after multiple rounds of infection which would lead to a strong decrease in overall virus production. In contrast to this expectation, the variants replace wild-type HBV in patients only to a certain extent and data on viremia (Wakita *et al.,* 1991; Günther *et al.,* 1996a; Tsubota *et al.,* 1998) do not indicate a simultaneous decrease in virus replication, both of which argue for an equilibrium between accumulation of variants and virus production *in vivo*. Furthermore, it is a specific feature of C gene deletion variants that they accumulate under immunosuppression (Günther *et al.,* 1996a) and disappear when the immune response is activated on cessation of immunosuppres-

FIG. 7. (A) Representative Southern blot analysis of particle-associated progeny DNA produced after cotransfection of variant or control with WT-b in different ratios. The marker contains a 3.2-kb double-stranded (ds) and a 3.2-kb single-stranded (ss) fragment. The positions of these fragments are indicated at the right. Note that the ss HBV DNA runs in some lanes at different positions due to variation of salt concentrations in individual samples. (B) Level of progeny HBV DNA in cytoplasm (solid line) and supernatant (dashed line) produced after cotransfection of variant with WT-b. The levels were standardized with the corresponding HBsAg values and plotted versus the percentage of variant in the transfected DNA mixture. Top: overall production of progeny DNA as determined by Southern blot (mean and standard deviation; 20% variant, $n = 3$; 50% variant, $n = 1$; 66% variant, $n = 2$; 100% variant, $n = 1$). Bottom: progeny DNA synthesized proportionately by variant and WT-b, calculated with the level of overall progeny DNA and the corresponding enrichment factor as described under Materials and Methods. (C) Level of progeny DNA produced proportionately by variant and WT-b in relation to the corresponding amount of transfected DNA (black bars, cytoplasm; shaded bars, supernatant).

sive treatment or seroconversion to anti-HBe (Günther et al., 1996a; Marinos et al., 1996). This points to the immune response to counteract or prevent accumulation of these variants. According to the variant-wild-type co-transfection data, accumulation of deletion variants in the nucleus may result in increased progeny DNA synthesis. If this is accompanied by an increase of the ccc DNA pool and viral protein synthesis, these cells could be more susceptible to T cell or cytokine-mediated killing. This scenario would be consistent with the severe liver disease—up to hepatic failure—that is seen in some patients infected with C gene deletion variants (Uchida et al., 1994; Günther et al., 1996a; Marinos et al., 1996; Zoulim et al., 1996).

In conclusion, this study demonstrates that HBV with deletions in the C gene has the capacity for enhanced replication which determines its interaction with wild-type HBV and has implications for selection, immune recognition, and virulence of these variants.

MATERIALS AND METHODS

Patients

Patients I, D, and G were renal transplant recipients under long-term immunosuppression (Günther et al., 1996a). Patient D selected C gene deletion variants 1 year before he died from end-stage liver disease; patient G was infected by C gene deletion variants while the liver disease decompensated; and patient I with chronic hepatitis lost C gene deletion variants upon cessation of immunosuppressive treatment and had no worsening of liver disease. Serum samples of patient I were taken at the beginning of follow-up and those of patients D and G at the end of follow-up. Patient FT was an immunocompetent patient with chronic active hepatitis infected with C gene deletion variants (Marinos et al., 1996). All patients were positive for HBsAg and HBeAg.

Construction of plasmids

Serum HBV DNA was purified by proteinase K digestion and phenol–chloroform extraction. The C gene was amplified for 30 cycles with Taq polymerase and primers 1767[+] (positions 1767–1788 starting from the EcoRI site, TTTGTACTAGGAGGCTGTAGGC) and 2510[−] (2510–2487, AGGTACAGTAGAAGAATAAAGCCC), cloned into vector pCR II (Invitrogen, Leek, The Netherlands), and sequenced. Representative C gene fragments with deletions were exchanged with the corresponding C gene fragment of a monomeric HBV wild-type genome (Galibert et al., 1979) integrated in plasmid pHBV-SapI (referred to as WT-b). For subcloning, C gene fragments with deletions of 90, 24, or 120 nucleotides, derived from patients I, D, and G, respectively, were amplified for 15 cycles with Pwo polymerase (Boehringer Mannheim,

Mannheim, Germany), 0.5 μg plasmid DNA template, and 1 μM primer 1821[+] (1821–1841, CCGGAAAGCTT-GAGCTCTTCTTTTTCACCTCTGCCTAATCA, HindIII site for cloning underlined) and primer 2353[−] (2353–2314, TCGTCTAACAACAGTAGTCTCCGGAAGTGTTGATAGG-ATA, BspEI site for cloning underlined). A C gene with a deletion of 144 nucleotides, derived from patient FT, was amplified using primers 1821[+] and 2428[−] (2428–2405, AGATCTTCTGCGACGCGGCGATTG, BsaBI half-site underlined). The PCR products amplified with primers 1821[+] and 2353[−] were cleaved at the BspEI site of primer 2353[−] and were cloned into SmaI–BspEI-digested pHBV-SapI, resulting in hybrid HBV genomes ΔC-90, ΔC-24, and ΔC-120. A wild-type hybrid genome (WT-a) with a C gene of genotype A, as well as a control genome (WT-core[−]) with a C gene of genotype A that is defective due to a nucleotide deletion, were constructed similarly. The PCR products amplified with primers 1821[+] and 2428[−] were cleaved at the HindIII site of primer 1821[+] and were cloned into HindIII–BsaBI-digested pHBV-SapI, resulting in hybrid genome ΔC-144. Genome ΔC-144pol[−] is identical to ΔC-144, but contains a nucleotide deletion created artificially at the BsaBI cloning site. This leads to a frame shift in the P gene and renders the hybrid genome defective in polymerase expression. Correct insertion and sequence of the C gene in the resulting hybrid genomes was ascertained by sequencing. Plasmid pCMV core contains the genotype D wild-type core protein sequence (Galibert et al., 1979) under control of the CMV promoter (kindly provided by H. Schaller, Heidelberg).

Transfection of HBV DNA

Plasmids were prepared by the Qiagen procedure (Qiagen, Hilden, Germany) and additionally purified with RNase A and proteinase K (Boehringer Mannheim) to remove traces of contaminating RNA and protein. Linear HBV monomers with SapI-sticky ends were released from pHBV-SapI and its derivatives by cleavage with 1.5 U SapI/μg DNA (New England Biolabs) for 12 h. Without further purification, 3 to 5 μg cleaved DNA [which is in the linear range of the dose–response curve (Günther et al., 1995)] were transfected alone or mixed with 2 to 4 μg pCMV core according to the calcium phosphate precipitation method. HuH7 cells were plated at a density of 1.2×10^6 cells per 50-mm-diameter petri dish. Medium was changed 1 day after transfection, and cells were harvested 2–3 days later. Transfection efficiency was measured by cotransfection of 1 μg of reporter plasmid expressing secreted alkaline phosphatase (SEAP) and determination of SEAP enzymatic activity in the cell culture supernatant. SEAP activity was similar for all variants. HBsAg and HBeAg in the medium were assayed using commercially available kits (AxSYM, Abbott).

Purification of HBV DNA from intracellular and extracellular core particles

Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in 1 ml lysis buffer (50 mM Tris–HCl [pH 7.4], 1 mM EDTA, 1% NP-40) per 50-mm-diameter petri dish. The lysed cells were vortexed and allowed to stand on ice for 15 min. Nuclei were pelleted by centrifugation for 1 min at 14 krpm in a tabletop centrifuge (Eppendorf 5415C). A fraction (25–50%) of the supernatant was adjusted to 10 mM $MgCl_2$ and treated with 100 μg DNaseI/ml for 30 min at 37°C. The reaction was stopped by adding EDTA to a final concentration of 25 mM. Proteins were digested with 0.5 mg of proteinase K per ml–1% SDS for 2 h at 37°C. Nucleic acids were purified by phenol–chloroform (1:1) extraction and ethanol precipitation.

The cell culture medium was clarified by centrifugation at 8 krpm for 30 min (Sorvall SS-34 rotor). Four milliliters of medium was layered on top of a 1-ml 20% sucrose cushion in 10 mM Tris–HCl (pH 7.4)–1 mM EDTA–100 mM NaCl and centrifuged at 45 krpm for 4 h (Beckmann SW50.1 rotor). The pellet was resuspended in 500 μl of 50 mM Tris–HCl (pH 8.0) and treated as described above for the isolation of HBV DNA from cytoplasmic core particles.

Purification of ccc HBV DNA from nuclei by alkaline lysis

Pelleted nuclei were resuspended in 100 μl of solution I (50 mM glucose, 25 mM Tris/HCl [pH 8.0], 10 mM EDTA), mixed with 200 μl of solution II (0.2 N NaOH, 1% SDS), and incubated for 5 min at room temperature. After the addition of 150 μl of solution III (3 M K acetate, 11.5% acetic acid) and incubation for 10 min on ice, nuclear debris were pelleted at 14 krpm in a tabletop centrifuge. Nucleic acids in the supernatant were purified by phenol–chloroform (1:1) extraction and ethanol precipitation.

Preparation of total RNA

Cells were washed twice with ice-cold PBS and lysed in 5 ml of guanidinium lysis buffer (2 M guanidinium thiozyanate, 12 mM Na citrate, 0.2 M Na acetate, 50% [vol/vol] phenol [water-saturated], 0.4% [vol/vol] β-mer-captoethanol) per 50-mm-diameter petri dish. After the addition of 500 μl of chloroform and 15 min of incubation on ice, the lysates were centrifuged for 20 min at 11.5 krpm (Sorvall SS-34 rotor). The RNA in the aqueous phase was precipitated with isopropanol, pelleted by centrifugation, washed with ethanol, and dissolved in water.

Southern and Northern (RNA) blot analysis

DNAs isolated from viral particles were separated on a 1.5% agarose gel and 20 μg of total RNA was analyzed in a 1.5% agarose–formaldehyde gel. Nucleic acids were blotted onto Hybond N nylon membranes (Amersham) and hybridized with ^{32}P-labeled fragments synthesized with full-length HBV DNA or β-actin cDNA as a control. The probe was generated using a random-primed labeling kit (Amersham). Autoradiography was analyzed with FUJIX BAS 2000 (Fuji, Tokyo, Japan).

Immunoblot analysis

Cells were washed with ice-cold PBS, collected in 1 ml of PBS, and pelleted at 2 krpm in a tabletop centrifuge. The cell pellet was lysed in 5 vol of SDS loading buffer, boiled for 5 min, sonicated, and centrifuged. For detection of core protein, total cell lysate was separated in an SDS–17.5% polyacrylamide gel. The proteins were transferred in Towbin buffer to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) and detected using polyclonal rabbit anti-HBc-83 (Bichko et al., 1993) (dilution 1:5000). After separation of the cell lysate in a 10% gel and blotting to nitrocellulose membrane (Schleicher & Schuell, Germany), HBV polymerase and pre-S1/2 proteins were detected using monoclonal antibodies anti-Pol 3.12F (unpublished) (diluted 1:10) and a mixture of anti-pre-S2 2F12 and 1-9D1 (each diluted 1:40; kindly provided by W. Gerlich, Gießen, Germany), respectively. For protein detection, three rounds of incubation with primary and secondary antibody were performed before detection by chemiluminescence. The image was digitized using a scanner.

RFLP assay to measure enrichment of variants

Following cotransfection of variant genomes with WT-b, HBV DNA was isolated from nuclei as well as from intra- and extracellular particles and amplified with the Expand high fidelity PCR assay (Boehringer Mannheim) and 0.2 μM primers 1645[+] (1645–1669, IRD-labeled-AGGTCTTACATAA-GAGGACTCTGGG) and 1977[−] (1977–1952, CGGTCCAGC-CCGGCGTCGACGAAGGAAAGAAGTCAGAAGGCAAAAA, TaqI site for cleavage control underlined). The PCR was run for 40 cycles with 1 min at 94°C, 1.5 min at 55°C, and 1.5 min at 72°C in a Robocycler (Stratagene). The PCR products were purified (QIAquick PCR purification kit, Qiagen) and 10% thereof were digested with 10 U TaqI in a 10-μl assay. An aliquot was denatured and resolved in a 5% polyacrylamide–7 M urea gel on an automated sequencer (LI-COR, Lincoln, NE). Separated restriction fragments were detected and quantified via the fluorescence-dye-labeled primer and enrichment was calculated as follows: enrichment factor = (signal of variant in cytoplasm or supernatant × signal of WT-b in nucleus)/(signal of variant in nucleus × signal of WT-b in cytoplasm or supernatant).

Calculations

The level of progeny variant and progeny WT-b DNA ($P_{\Delta C}$ and $P_{WT\text{-}b}$) produced proportionately following co-

transfection of both virus DNAs was calculated with the percentage of variant and WT-b DNA in the transfection mixture ($T_{\Delta C}$ and T_{WT-b}, respectively; both sum up to 1.0 [100%]), the corresponding, experimentally determined level of total progeny DNA (P_{total}), and the corresponding, experimentally determined enrichment factor (f). The ratio of progeny variant to progeny WT-b DNA is described by the equation $P_{\Delta C}/P_{WT-b} = fT_{\Delta C}/T_{WT-b}$, and the sum of progeny variant and progeny WT-b DNA is described by the equation $P_{WT-b} + P_{\Delta C} = P_{total}$. Both equations were combined to determine the level of progeny DNA of variant and WT-b: $P_{\Delta C} = P_{total}/(1 + T_{WT-b}/T_{\Delta C}f)$ and $P_{WT-b} = P_{total}/(1 + fT_{\Delta C}/T_{WT-b})$. The level of progeny DNA in relation to the corresponding amount of transfected DNA was calculated as $P_{\Delta C}/T_{\Delta C}$ and P_{WT-b}/T_{WT-b}.

Image processing and quantitative data evaluation

Digitized autoradiography, chemiluminescence, and fluorescence signals were quantified using TINA software (Raytest, Straubenhardt, Germany). For presentation, brightness and contrast of the images were adjusted with Corel Photo Paint software (Corel Corporation, Ottawa, Canada).

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