Reduced antigen production by hepatitis B virus harbouring nucleotide deletions in the overlapping X gene and precore-core promoter

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Hepatitis B virus (HBV) genomes with deletions in the precore-core (preC-C) promoter have been detected in HBV infections without serological markers. To address whether the mutations are responsible for the reduced production of virus antigens, either an 8 bp (8d, position 1763 to 1770) or a 20 bp (20d, 1753 to 1772) deletion was created in a wild-type (wt) HBV clone. Both mutations cause premature termination of the overlapping X ORF. When introduced into HepG2 cells, both mutants produced reduced amounts of HBsAg, HBeAg and HBeAg, but released the same or more virion-associated DNA compared with the wt. A co-transfection of the 20d mutant with a small amount of intact X gene resulted in a 3-fold increase of HBeAg production compared to transfection with either the 20d or wt alone. When the promoter region was cloned into CAT plasmids, the 8d preC promoter showed weak activity and its initiation site was shifted 6 to 10 bp downstream. The preC promoter activity of 20d was not detectable by CAT ELISA and 5'-RACE. The levels of C transcripts of both mutants were higher than that of the wt, and their start sites were not altered. Therefore, the deletions cause the reduction of HBsAg, HBeAg and HBeAg although the mutant viruses can still replicate in cultured cells. The reduction of HBeAg is due to both the reduced preC promoter activity and the defect in HBx. The reduction of HBeAg is due to the disrupted X gene, despite augmented C promoter activity.

Introduction

Hepatitis B virus (HBV) DNA with deletions in the precore-core (preC-C) promoter overlapping the X gene has been detected in a variety of hepatitis cases, including chronic hepatitis B (Feitelson et al., 1991, 1995a, b; Repp et al., 1992), HBsAg-negative post-transfusion hepatitis (Feitelson, 1994), HBV infection with no serological marker (Preisler-Adams et al., 1993, 1994) and anti-HBe seropositive infections (Kim et al., 1992; Laskus et al., 1994; Okamoto et al., 1994). Infection solely by a deletion mutant results in low titres of HBV DNA in serum. In contrast, HBV DNA titres vary among those infected with a quasispecies including both mutant and wild-type (wt) viruses (Okamoto et al., 1994).

The HBx transcription factor, a 154 amino acid (aa) product of the X gene, trans-activates gene expression from diverse viral and cellular genes (Twu & Schloemer, 1987; Trusnt et al., 1995), including HBV C and S (Nakatake et al., 1993; Spandau & Lee, 1988; Colgrove et al., 1989). It has been demonstrated that HBx lacking the carboxyl-terminal 24 aa cannot act as a transcriptional trans-activator (Runkel et al., 1993; Takada & Koike, 1994). This region is associated with most of the X gene deletion mutations detected in clinical samples. Therefore, deletions in this region are considered to be responsible for the reduced viral antigen production as well as several types of disease progression (for a review, see Feitelson, 1994).

The preC gene product, HBeAg precursor, is translated from 3-5 kb over-genome length transcripts, which initiate 22 to 28 bp upstream from the preC initiation codon (Weimer et al., 1987; Yaginuma & Koike, 1989). The precursor loses 19 amino-terminal aa and 36 (or 34 in several subtypes bearing a 6 bp shorter C gene) carboxyl-terminal aa for secretion from infected hepatocytes (Bruss & Gerlich, 1988; Jean-Jean et al., 1989). Although the biological function of HBeAg is still unclear, patients with the HBeAg marker generally have high

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levels of virus and higher rates of transmission than those who are anti-HBe-seropositive (Okada et al., 1976; Alter et al., 1976).

The C gene product, HBeAg, is translated from another 3·5 kb transcript, of which the 5′ end is 29 to 35 nucleotides further downstream than that of the preC mRNA, and in which the preC initiation codon is absent. This transcript also serves as the mRNA for both the DNA polymerase—reverse transcriptase and the pregenome (Mason et al., 1982; Summers & Mason, 1982; Seeger et al., 1986; Nassal et al., 1990; Ou et al., 1990). Thus, mutations affecting levels of C transcripts most likely play a role in pathology.

Transcription of the C mRNA is controlled by an upstream regulatory element and the basic C promoter (position 1643 to 1849) (Yaginuma & Koike, 1989; Yuh et al., 1992) as well as a negative regulatory element (1611 to 1634) (Lo & Ting, 1994). The function of this C promoter region in the transcription of overlapping preC mRNA has not been examined in detail.

In this study, it was demonstrated that the 8 bp (1763 to 1770, between the second and third AT-rich regions) and 20 bp (1753 to 1772, encompassing the first and second AT-rich regions) deletion mutations in the preC-C promoter of dimeric HBV DNA are responsible for the reduced production of HBsAg, HBeAg and HBeAg in transfected HepG2 cells. Also, it was found that the mutations cause remarkable reductions in preC promoter activity as well as a small enhancement of C promoter activity.

To construct pBasic.d5′ CAT, in which 38 bp were deleted from the 5′ UTR of the chloramphenicol acetyltransferase (CAT) gene of pCAT-Basic (Promega), part of the CAT ORF encompassing the region from the initiation codon to a unique Neol site was amplified by PCR using primers B077 (5′ GACCTCTAGCTAAAAATGGAGAAAAATCCA) and B078 (5′ CAGCACCTTGGCGCTTGGC), and ligated with the larger segment of Xbal-d and Neol-digested pCAT-Basic DNA. For p8d.d5′ CAT and p20d.d5′ CAT, a DNA fragment from 1630 to 1799 of each wt or mutated HBV DNA was amplified by PCR using primers B075 (5′ GAACCTCTAGCTAAAAATGGAGAAAAATCCA) and B068 (5′ TGCCATGCAAAATATAGCCTAAGC), each HBV DNA between positions 1630 and 1846 was amplified with primers B067 and B079 (5′ CAGACCTAAGATGTTGCGGAGGT), and then cloned into the SphI site, and the amplified products were cloned into the SphI site of pBasic.d5′ CAT (see Fig. 4A). For p8d.79.CAT and p20d.79.CAT, each HBV DNA between positions 1630 and 1846 was amplified with primers B067 and B079 (5′ CAGACCTAAGATGTTGCGGAGGT), digested with SphI and XbaI, and then cloned into the SphI–XbaI digest of the pCAT-Basic vector.

**Methods**

- **Plasmids carrying a tandem dimer of whole-genome HBV DNA.** Plasmid pYW8d carries an HBV DNA with an 8 bp deletion (8d) from 1763 to 1770 (Fig. 1). This was produced by PCR-based site-directed mutagenesis (Landt et al., 1990) of plasmid pPYW310, a pUC119 having a 1·182 bp complete HBV genome (subtype ayw) at the SphI site. The HBV DNA was cloned from the serum of an HBeAg-positive carrier (Okamoto et al., 1990). The two first-step PCRs were performed with a forward primer B026 (5′ GACAACCTCTAGCTAAAAATGGAGAAAAATCCA) and a mutated reverse primer B058 (5′ AGGTTGACCTTGGTGTTGCTTCTCC), and a reverse primer B046 (5′ TGCTCAGGAGGAACTCAGGCT) and a mutated forward primer B057 (5′ GATGAGTTAAATACTAGGGAGGT), and then cloned into the SphI–XbaI digest of pCAT-Basic DNA. For p8d.d5′ CAT, in which 5′ UTR of the chloramphenicol acetyltransferase (CAT) gene of pCAT-Basic (Promega), part of the CAT ORF encompassing the region from the initiation codon to a unique Neol site was amplified by PCR using primers B077 (5′ GACCTCTAGCTAAAAATGGAGAAAAATCCA) and B078 (5′ CAGCACCTTGGCGCTTGGC), and ligated with the larger segment of Xbal-d and Neol-digested pCAT-Basic DNA. For p8d.d5′ CAT and p20d.d5′ CAT, a DNA fragment from 1630 to 1799 of each wt or mutated HBV DNA was amplified by PCR using primers B075 (5′ GAACCTCTAGCTAAAAATGGAGAAAAATCCA) and B068 (5′ TGCCATGCAAAATATAGCCTAAGC), each HBV DNA between positions 1630 and 1846 was amplified with primers B067 and B079 (5′ CAGACCTAAGATGTTGCGGAGGT), digested with SphI and XbaI, and then cloned into the SphI–XbaI digest of the pCAT-Basic vector.

- **Transfection.** HepG2 human hepatoma cells (Aden et al., 1979) were cultured in 60 mm dishes containing DMEM supplemented with 10% calf serum (CS). Recombinant plasmids were introduced into the cells using polycationic lipids (LipofectAMINE, Gibco). For triplate transfections, 15·0 μg of plasmid carrying HBV genomes and 3·0 μg of pTKGH (Selden et al., 1986) were mixed with 90 μl of the lipid solution in 1·2 ml of reduced serum medium (Gibco). The mixture was incubated at 22 °C for 30 min, then diluted with 4·8 ml of the reduced serum medium. Two ml of 10% diluted mixture per dish was overlaid on the cells. After 6 h of incubation at 37 °C, 2·0 ml of DMEM containing 20% CS was added, and the dishes were incubated for a further 12 h. Then the culture medium was replaced with 4 ml of fresh DMEM containing 10% CS.

- **ELISA of HBV antigens, human growth hormone (hGH) and CAT.** For ELISA of the cell lysates for HBV antigens, cells were rinsed with 5 ml of PBS three times, scraped into 1 ml of PBS and pelleted by brief centrifugation. The cell pellet was suspended in 200 ml of 0·25 M Tris–HCl, pH 7·5, and stored at —70 °C. For ELISA, an equal volume of 2 × lysis solution consisting of 150 mM NaCl, 20 mM EDTA, pH 8·0, 1 mM PMSF (Sigma) was added to the suspension, and the mixture was vortexed for 15 s and centrifuged at 12,000 g for 3 min. Part of the supernatant was then diluted to an appropriate concentration to fit within the linear range of light absorbance. HBsAg was tested using anti-HBs substrate (Sigma).

For CAT-ELISA, HepG2 cells in a 60 mm dish were transfected with 5 μg of CAT plasmid along with 1μg of pTKGH with polycationic lipids. Two days after transfection, the cells were harvested and extract was prepared as described for the HBeAg ELISA. The CAT polypeptide was quantified with a CAT-ELISA kit (Boehringer Mannheim) and o-phenylenediamine (OPD) substrate (Sigma).

- **Southern blotting.** To isolate encapsidated viral DNA, 4 ml of culture medium was centrifuged at 273,000 g for 3 h. The pellet was suspended in 1 ml PBS, overlaid on 3·5 ml of 20% sucrose–PBS, and centrifuged at 273,000 g for 3 h. Tris–HCl, pH 8·0, CaCl₂ and staphylococcal nuclease (Sigma) were added to the resulting pellet to provide final concentrations of 10 mM, 5 mM and 1 U/ml in 1 ml, respectively. The
reaction mixture was diluted to 0.5% (w/v) in SDS and 0.2 mg/ml in proteinase K, and further incubated at 55 °C for 2 h. DNA was extracted with phenol and chloroform, and precipitated with ethanol. The DNA was then completely digested with DpnI, subjected to electrophoresis on 1% agarose gel and blotted onto a nylon filter membrane. Hybridization was carried out with PCR-amplified HBV DNA (spanning positions 747 to 1823 and 2413 to 3182/1 to 197 of pPYW310) labelled with [32P]dCTP, and the filters were washed under highly stringent conditions. Radioactivity of specific bands was counted with a BAS 2000 image analyser (Fuji film).

**PCR and restriction fragment length polymorphism of progeny virus DNA of the mixed transfection.** DNA was extracted from the culture medium of cells co-transfected with p2W20d and p2UCP as described above, and contaminating plasmid DNA harbouring the HBV genome was digested extensively with the methylation-sensitive restriction endonuclease DpnI. Thus, the DNA replicated in transfected cells, but not plasmid DNA, could be amplified by PCR using primers which anneal to the region outside the DpnI sites. The DpnI sites were located at positions 1401 and 1985 (see Fig. 3A). HBV DNA from position 1260 to 2046, encompassing the preC-C promoter, was amplified by PCR (25 cycles) using primers B26 and B46, digested with StyI and electrophoresed in 5% agarose gel. StyI sites were located at positions 1372, 1642 and 1882. The amplified progeny DNA of p2UCP and p2W20d contained 240 and 220 bp StyI fragments, respectively.

**Anchored 5’ RACE.** Cells in a 60 mm dish were harvested 48 h after transfection with one of the CAT plasmids. Total RNA was isolated and treated extensively with DNase I. The quantities of both the preC and C mRNA were determined by anchored-RACE (Edwards et al., 1990). cDNA was synthesized with primer B78 using reverse transcriptase and treated extensively with DNase I. The cDNA was ligated with a excess primers and dNTP were removed by passage through an agarose column (Chromaspin-100, Funakoshi) and the cDNA was ligated with a anchored 5’ phosphorlated and 3’ aminated 46-mer anchor deoxyoligonucleotide, B100 (5’ PO4-GACATCTGACTGATCCACTGAATCTTTAGTGAGGTTATGGCC-NH2), using T4 RNA ligase (Takara). After a 24 h ligation reaction in 10 μl of reaction mixture at 16 °C, half of the mixture was used for the first PCR with primers B101 (5’ GGCAATACCCTCCTCATAAGG) and UT3 (5’ AAGGCGGAAATATCCAGC), which anneal with the anchor and cDNA, respectively. The EcoRl- and BamHI-digested PCR products were cloned into pUC19, and the 3’ end of the cDNA was determined by dyeoxy nucleotide sequencing (Amersham).

**Results**

**Reduced viral antigen production and replication of the preC-C promoter deletion mutant**

To clarify how the previously reported nucleotide deletions in the preC-C promoter are associated with the marker-negative phenotype, homogeneous tandem dimers of the whole HBV genome with deletions in the preC-C promoter region were created (Fig. 1A). p2W8d has a homogeneous tandem dimer of HBV DNA (from wt p2UCP) bearing an 8 bp deletion (8d) from position 1763 to 1770 located between the second (1757 to 1762) and third (1789 to 1795) AT-rich regions in the preC-C promoter. This deletion has been found in several HBeAg-negative infections (Repp et al., 1992; Laskus et al., 1994; Feitelson, 1994; Okamoto et al., 1994). p2W20d has a homogeneous tandem dimer of HBV DNA bearing a 20 bp deletion (20d) from 1853 to 1772. This deletion has been also found in several HBeAg-negative infections (Okamoto et al., 1994). 20d disrupts the first (1752 to 1755) and second AT-rich regions of the preC-C promoter. Both 8 bp and 20 bp mutations prematurely terminate the X ORF at codons 135 and 131, respectively (Fig. 1B).

When introduced into HepG2 cells, p2W8d produced HBsAg and HBeAg at levels detectable by ELISA. However, the quantities of HBsAg and HBeAg 72 h after transfection were 26% and 15%, respectively, of the amounts produced...
Table 1. HBV antigen titres in cell extracts and culture medium of HepG2 cells transfected with either a wild-type or a mutated HBV genome

<table>
<thead>
<tr>
<th></th>
<th>hGH in medium</th>
<th>HBsAg in medium</th>
<th>HBeAg in medium</th>
<th>HBeAg in cell extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>p2UCP</td>
<td>788*</td>
<td>1502 (204 ± 26)†</td>
<td>519 (71 ± 8)</td>
<td>1331 (176 ± 5)</td>
</tr>
<tr>
<td>p2W8d</td>
<td>1030</td>
<td>545 (54 ± 12)</td>
<td>290 (29 ± 11)</td>
<td>290 (26 ± 7)</td>
</tr>
<tr>
<td>p2W20d</td>
<td>839</td>
<td>619 (76 ± 14)</td>
<td>484 (61 ± 12)</td>
<td>98 (8 ± 2)</td>
</tr>
<tr>
<td>ΔC</td>
<td>738</td>
<td>1640 (235 ± 22)</td>
<td>10</td>
<td>34</td>
</tr>
<tr>
<td>Mock</td>
<td>52</td>
<td>35</td>
<td>10</td>
<td>32</td>
</tr>
</tbody>
</table>

* The average light absorbance in ELISA of triplicate transfections.
† The light absorbance value for each antigen was converted to the absolute value with the standard curve, divided by the absolute amount of hGH in each case, and the average ± 1SD of the resulting triplicate relative values given in parentheses.

with p2UCP (Table 1). The mutation also reduced the amount of HBeAg, but appear not to affect production of progeny virus in vitro.

Induction of viral antigen production of the mutated HBV DNA by co-transfected HBV DNA bearing the intact X gene

Most clinical cases in which only a deletion mutant was detected have been reported to harbour low HBV DNA titres in their serum, whereas the titres vary among those infected with both mutant and wt viruses. To address how the mutants interact with a wt virus, trans-complementation experiments were performed. When p2W20d was co-transfected with a limited amount of p2UCP (ratio 9:1), production of HBsAg, HBeAg and HBeAg was enhanced 5-, 5- and 2.5-fold, respectively, over the amounts produced with p2W20d alone (transfection numbers TF 1 and 2 in Table 2). The HBeAg titre of this mixed transfection was higher than that of p2UCP alone (TF 4). Increased antigen production was also observed when p2W20d was co-transfected with a small amount of ΔC, which has defective preC and C ORFs but bears intact X, preS-S and pol genes (TF 1 and 3).

Restriction fragment length polymorphism of the PCR products of the encapsidated (nuclease S7-resistant) and DpnI-resistant HBV DNA purified from the culture medium showed that the mutant progeny predominated (Fig. 3). Thus, augmented viral antigen production in the co-transfection is predominantly due to increased gene expression of deletion mutants. Amplification of co-transfected wt or ΔC DNA template is unlikely to be the cause of enhanced antigen production.

CAT ELISA and 5’ RACE of the mutant promoter region

Both preC and C promoter activities were quantified using the CAT gene as a reporter in order to eliminate any possible contribution by the truncated HBx protein. pWI.d5’CAT and...
Table 2. HBV antigen titres in cell extracts and culture medium of HepG2 cells co-transfected with 20 bp deletion mutant and HBV DNA(s) bearing an intact X gene

<table>
<thead>
<tr>
<th>TF no.</th>
<th>hGH in medium</th>
<th>HBsAg in medium</th>
<th>HbcAg in cell extract</th>
<th>HBeAg in medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) p2W20d</td>
<td>997*</td>
<td>201 (19 ± 13)†</td>
<td>702 (72 ± 6)</td>
<td>73 (2 ± 2)</td>
</tr>
<tr>
<td>(2) p2W20d + p2UCP (9:1)‡</td>
<td>681</td>
<td>680 (105 ± 15)</td>
<td>2189 (346 ± 16)</td>
<td>186 (218 ± 18)</td>
</tr>
<tr>
<td>(3) p2W20d + ΔC (9:1)</td>
<td>467</td>
<td>357 (80 ± 27)</td>
<td>1229 (292 ± 20)</td>
<td>196 (357 ± 21)</td>
</tr>
<tr>
<td>(4) p2UCP</td>
<td>886</td>
<td>1042 (122 ± 11)</td>
<td>675 (78 ± 10)</td>
<td>525 (575 ± 24)</td>
</tr>
<tr>
<td>(5) p2UCP + p2W20d (9:1)</td>
<td>960</td>
<td>1252 (153 ± 20)</td>
<td>860 (91 ± 8)</td>
<td>723 (740 ± 28)</td>
</tr>
<tr>
<td>Mock</td>
<td>57</td>
<td>27</td>
<td>29</td>
<td>50</td>
</tr>
</tbody>
</table>

* The average light absorbance in ELISA of triplicate cultures.
† The light absorbance value for each antigen was converted to the absolute value with the standard curve, divided by the absolute amount of hGH in each case, and the average ± 1SD of the resulting triplicate relative values given in parentheses.
‡ Molar ratio.

Fig. 3. Restriction fragment length polymorphism of progeny virus DNA in culture medium of cells transfected with either a single mutant or a combination of two mutants as shown in Table 2. (A) DNA was obtained from the ultracentrifuge-purified S7 nuclease-treated virions and further treated with DpnI. The preC-C promoter region of DNA generated in cells was PCR amplified. The products were digested with StyI and electrophoresed in 5% agarose gel. The bands at 220 bp and 240 bp represent progeny DNA of p2W20d and p2UCP, respectively. (B) The 20 bp-deleted genome (underlined) contributed the majority of progeny virus in co-transfections with p2W20d and p2UCP in 9:1 ratios.

its derivatives harbour either a wt or a mutated 170 bp fragment from positions 1630 to 1799. The authentic 22 bp 5′ UTR for the preC gene was replaced with a hybrid 5′ UTR containing 8 bp of the preC gene and 14 bp of the CAT gene. Therefore, only transcripts that are initiated upstream from the CAT initiation codon (the preC initiation codon equivalent) can be translated. Thus, the p.d5′CAT series tests preC promoter activities (Fig. 4A). Transfections of HepG2 cells with plasmids bearing mutated preC promoter regions resulted in less CAT antigen production (p8d.d5′CAT, 20%; p20d.d5′CAT, 10%) than in those transfected with the wt construct (pWI.d5′CAT) (Fig. 4B).

The p.79.CAT series carries a mutated 216 bp fragment from position 1630 to 1846 (Fig. 4A). The inserted promoter fragments contain the initiation codon of the HBcAg precursor, but this reading frame terminates at the TAA codon 13 bp upstream from the CAT initiation codon. Thus, the titre of CAT in HepG2 should reflect only the C transcript equivalents, which start downstream from the preC initiation codon at 1814. Transfection with the mutated plasmids, p8d.79.CAT and p20d.79.CAT produced the same amount of CAT as did pWI.79.CAT (Fig. 4C).

To determine if differences in CAT titre are due to differences in the quantity of transcript, the length of 5′ UTR, or both, semiquantitative 5′ RACE was performed. Agarose gel electrophoresis of RACE products of CAT mRNA of p8d.79.CAT and p20d.79.CAT showed markedly reduced preC mRNA-equivalents (Fig. 5A, B). Moreover, 4-fold increases in C mRNA equivalents of both mutants were found (Fig. 5A, B). The RACE product for the preC transcript of p8d.79.CAT was slightly shorter than that of pWI.79.CAT (Fig. 5B). Nucleotide sequencing of preC equivalent RACE products of p8d.79.CAT showed 6 to 10 bp downstream shifts of its initiation site (Fig. 5C). Attempts to clone the preC RACE products of p20d.79.CAT, which were not visible in an ethidium bromide staining, were unsuccessful. The initiation sites of C transcript-equivalents of the wt and both mutant constructs were at the same position, 1817 (Fig. 5C).
Discussion

It has been demonstrated that an HBx deleted at the carboxyl-terminal end from aa 130 does not function as a trans-acting transcriptional activator (Takada & Koike, 1994). In this study, co-transfection of p2W20d with the intact X gene (either wt or ∆C) showed enhanced production of antigens by p2W20d (Table 2). The wt as well as mutated preC and C promoter activities in CAT plasmids (p.d5′CAT and p.79.CAT series) were enhanced by co-transfected p2UCP (unpublished observation). Therefore, the disruption of the X gene ORF, rather than an augmentation of the C promoter basal activity, causes the reduction of HBcAg production. Reduction of HBeAg is due to both the altered X gene and reduced preC promoter activity. Also, the shortened 5′ UTR of preC mRNA of p2W8d may contribute to reduced HBeAg production by lessening translation efficiency.

Fig. 4. Activities of mutated preC-C promoters determined by CAT-ELISA 3 days after transfection of HepG2 cells with preC-C promoter-CAT constructs. (A) Structures of CAT-plasmids harbouring a preC-C promoter fragment. HBV DNA is represented by thick lines, the original CAT plasmid DNA by horizontal dashed lines, open reading frames by boxes, transcriptional start sites of the preC and C mRNAs of p2UCP by large arrows, and the 5′ UTR of the preC transcripts by small arrows. (B) CAT-ELISA titres of cells transfected with the p.d5′CAT series, representing the preC promoter activity. (C) p.79.CAT series, representing the C promoter activity. pTKGH was co-transfected as an internal control for transfection efficiency; the A492 minus the background of the CAT-ELISA was divided by the A492 minus the background of hGH-ELISA, and the result given in arbitrary units. The average and 1 SD of the values from triplicate transfections are shown by the black bars and error bars, respectively.

Fig. 5. Anchored 5′ RACE of CAT transcripts after transfection of HepG2 cells with p.79.CAT plasmids. (A) Schematic diagram of 5′ RACE. (B) The semi quantitative RACE products were electrophoresed in 5% agarose gel and visualized with ethidium bromide. (C) Nucleotide sequences of the RACE products. RACE products were digested with BamHI and EcoRI, electrophoresed in 5% agarose gel, and the bands with sizes between 70 bp and 140 bp were excised and ligated with plasmid vectors. Three clones each for preC and C transcriptions were picked up and the nucleotide sequences were determined. The resulting 3′ ends of the RACE products indicating the 5′ ends of transcripts are indicated by dots. Underlined sequences represent AT-rich regions in the promoter.
This study provides evidence that the 8d and 20d mutations are responsible for the reduced production of HBsAg, HBeAg and HBeAg, as predicted by clinical observations. Unexpectedly, however, equal or increased amounts of virion-associated DNA were detected in the culture medium of the mutants. Although C protein production is reduced by the mutations, it may be sufficient to encapsidate all of the transcribed pregenomes. The inhibitory role of the HBeAg precursor in the proliferation process has been shown by Lamberts et al. (1993). In the present experiment, the reduction in HBeAg might have facilitated the encapsidation and/or reverse transcription of the mutant pregenomes.

The 8d mutation down-regulated preC promoter activity and shifted its initiation site 6 to 10 bp downstream. The 20d mutation down-regulated preC promoter activity to an undetectable level (Fig. 4B). These results suggest that the second AT-rich region from 1757 to 1762 determines the preC mRNA initiation sites. This differs from a previous report describing the initiator function of the third AT-rich region from 1789 to 1795 for preC transcription (Chen et al., 1995). The observed difference in the importance of the third AT-rich region might be due to difference in plasmid construction. In this study, the basic preC-C promoter region was cloned in a CAT-basic plasmid, while Chen et al. (1995) used only the third AT-rich region and it was cloned downstream of a strong exogenous enhancer. Since the preC initiation sites appear more varied when RNA was isolated from cells transfected with the whole genome (unpublished observation), the mechanism controlling preC transcription initiation may be more complex than initially suggested by pre-C promoter studies.

C promoter activity was upregulated to a small extent by both deletions, and no alteration of the initiation site was observed (Fig. 4B). Thus, it appears that the C transcription start site is determined by the third AT-rich region, as demonstrated by Chen et al. (1995). The upstream transcripts including C itself, pre-S, pre-X and the nearest preC can competitively inhibit the formation of the transcriptional complex at the C transcription start point (promoter occlusion) (Schaller & Fischer, 1991). Thus, it appears reasonable to speculate that the reduction in preC transcription caused by the mutations favoured the transcription of the C gene.

Reduced production of HBsAg, HBeAg and HBeAg might enable the mutant viruses to escape immunodetection. Such mutants might have emerged in a quasispecies infection: either an acute infection with both wt and mutants at the same time, or a primary infection with a wt followed by chronic infection with its unmutated and mutant progeny. The mutant virus might be able to replicate and be selected while the host was acquiring immune competence to the viral proteins. Whether such a deletion mutant alone is infectious is not known. HBx has been demonstrated to be indispensable for the establishment of woodchuck hepatitis virus (WHV) infection of woodchuck (Chen et al., 1993; Zoulim et al., 1994), although the amount of WHV genome has not been evaluated with a sensitive PCR. In contrast, the existence of HBV carriers who are negative for any virus marker (Feitelson, 1994) suggests that the deletion mutant alone could establish an infection. Thus, further studies are needed to address the impact of these mutant viruses in the development of hepatitis.

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